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ASPECTS OF HONEY QUALITY: CRYSTAL CONTROL IN LIQUID HONEY; ANALYSIS OF FUMAGILLIN AND ITS DECOMPOSITION PRODUCTS BY HPLC AND ELISA.

BY

© HANAA IBRAHIM ASSIL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

FOOD CHEMISTRY

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA

FALL 1991.

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ISBN 0-315-70109-9

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Degree:	DOCTOR OF PHILOSOPHY
Year this degree granted:	FALL 1991

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ASPECTS OF HONEY QUALITY: CRYSTAL CONTROL IN LIQUID HONEY; ANALYSIS OF FUMAGILLIN AND ITS DECOMPOSITION PRODUCTS BY HPLC AND ELISA submitted by HANAA IBRAHIM ASSIL in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in FOOD CHEMISTRY.

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TO MY FATHER AND MY MOTHER

ABSTRACT

The first part of this research focused on the main factors that lead to crystallization of honey produced in Alberta, Canada. The resin HPLC column loaded with Pb^{++} cations (Aminex HPX-87P, BioRad) was useful in separation of the sugars (85 °C, water as eluent, RI detection), but slight decomposition of the monosaccharides was observed, confirming an earlier report in the literature. The resin column in the H^+ form (Rezex-H^+, Phenomenex) was operated at room temperature (0.01 N H_2SO_4 as eluent), so no hydrolysis of sucrose occurred, and the resolution of glucose, fructose and sucrose peaks was maintained. HPLC analysis of 41 raw honey samples produced in Alberta revealed that glucose content ranges from 30.9 - 39.9 % (average = 34.2 %) and fructose content ranges from 35.4 - 41.7 % (average = 38.6 %). The average fructose/glucose ratio was 1.13 (range = 0.95 to 1.24). HPLC analysis of 30 processed (blended) honey samples also showed that all the samples have approximately the same carbohydrate composition (average 34.1 % glucose, 37.7 % fructose) and consistent fructose/glucose ratio (average = 1.11), regardless of their crystallization properties. Differences in rate of crystal formation in the samples was found to correlate with the temperature at which the honey was allowed to flow into retail-sized containers. Samples filled while hot (> 45 °C) remained liquid for over a year. Loss of moisture on storage was also observed in the samples (about 1 % moisture per year).

The other focus of this research was the study of fumagillin (FA), an antibiotic fed to bees in winter. Two sensitive and effective methods were developed for analysis of FA in honey: HPLC and ELISA. A literature HPLC analysis method for FA was adapted to honey systems (Phenomenex IB-SIL 5 C_{18} column, 22 °C, eluent was acetonitrile : water : acetic acid 500 : 500 : 1.5, UV detection at 350 nm). Minimum sample preparation was needed and the detection limit was 100 ppb FA in honey. No fumagillin could be detected in honey samples obtained from a producer that uses fumagillin as medication for the bees.

To develop the ELISA, protein-FA conjugates were prepared by reaction of the acid functionality in FA with N-hydroxysuccinimide in presence of DCC (in DMF, 4 °C, 24 h) followed by reaction with the protein in phosphate buffer (pH 7.6, 4 °C, 24 h). An LPH-FA conjugate was injected into 2 rabbits while a BSA-FA conjugate was used for coating the ELISA plates. When the serum was diluted 500 times and the honey diluted to 1.0 g/mL in water, honey containing 20 ppb of FA gave B/Bo of about 0.5. Under these conditions, 13 different honey samples gave B/Bo > 0.7.

FA is thermally stable in honey in the dark. However, in presence of light, there is fast degradation of the polyene chain (about 70 % loss in the amount of FA in honey, detected by HPLC, within 8 days in daylight/fluorescent light). When FA was heated in 10 % ethanol in water (pH 3.5, 60 °C, 42 days), the more labile epoxide in the molecule was hydrolyzed to produce the dihydroxy derivative of FA. In light, rearrangement of the polyene chain gave a cyclic structure.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Peter Sporns, for his guidance and continous encouragement and support through the course of this work. My gratitude also goes to Dr. Lee Griffith and Hasmukh Sheth, for helpful discussions and advice at several stages of the research, and to Leonard Steele for his assistance in several technical difficulties. The staff of the spectral and analytical services in the Chemistry Department of the University of Alberta are thanked for their assistance in obtaining spectral data for characterization of compounds.

Finally, I would like to thank my husband, Mahmoud, and my son, Mohammed, for their endless support, encouragement and patience through all stages of this research.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
BSA-FA	BSA-fumagillin conjugate
BSA-FS	BSA-fumagillyl hemisuccinate conjugate
CI-MS	Chemical ionization mass spectrum
DCC	1,3-dicyclohexylcarbodiimide
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
EI-MS	Electron impact mass spectrum
ELISA	Enzyme-linked immunosorbent assay
FA	Fumagillin
FDCH	Fumagillin dicyclohexylamine salt
FS	Fumagillyl hemisuccinate
HPLC	High performance liquid chromatography
IR	Infrared
LPH	*Limulus polyphemus* hemolymph
LPH-FA	LPH-fumagillin conjugate
MS	Mass spectra
mp	Melting point
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing Tween 20
POSFAB-MS	Positive fast atom bombardment mass spectrum
TLC	Thin layer chromatography
TWEEN 20	Polyoxyethylene sorbitan monolaurate

CHAPTER 1

INTRODUCTION

Honey is the sweet viscous substance produced by the honey bee (*Apis mellifera*) from the nectar of plants (White, 1978). The US Food and Drug Act of 1906 defines honey as the nectar of plants gathered, modified and stored in the comb by honey bees. It is levorotatory and contains a maximum of 25% water, 0.25% ash, and 8% sucrose (Doner, 1977).

Honey is a natural product with a unique flavor. It was man's first sweetener before the discovery of cane and beet sugars. Although it is now in competition with a variety of glucose and high fructose syrups, it is still in high demand in today's market. Honey is mainly used as a spread on bread or crackers, as a sweetener in cereals and drinks, and as an ingredient in breads and cakes. It is also used in confectionery, preserves, syrups, cosmetics and tobacco industries. Honey can be consumed the form of a fermented product, mead, but this use has declined since the expansion of grape production and the increased popularity of wines. In ancient times, several medicinal qualities were attributed to honey. Although most of these attributes are now regarded as fictitious, honey is used as a carrier or vehicle in some medicinal syrups and as a binding agent in pills (Gojmerac, 1980). It is also effective as an antiseptic dressing for wounds, since its high osmotic pressure dehydrates most microorganisms, inhibiting their growth. Hydrogen peroxide produced in honey enzymatically is also toxic to pathogens (Crane, 1990). When taken with lemon juice, honey provides soothing relief from coughs and a sore throat.

Honey is an important commodity in many countries. China, Mexico and Argentina are, at present, the largest exporters of honey. In Canada, figures reported for 1984 indicate that the production of honey was 43,298 tones, 44 % of which was exported (Crane, 1990).

Honey is made by the honey bee from nectar, which is secreted by plant glands, called nectaries, in the form of an aqueous sugar-containing material. This raw nectar contains mainly sugars and water in varying concentrations, depending on the floral source. The sugars present are usually a mixture of sucrose, glucose (dextrose) and fructose (levulose). However, some nectars contain only sucrose, and some are a mixture of only glucose and fructose. The water contents also vary in different floral sources, and may be in the range between 20-95% (Gojmerac, 1980; Crane, 1990).

The honey bee ingests nectar through its mouth parts into the honey sac and gut. In the honey sac, the bee adds enzymes to the raw honey. The enzyme invertase (α-glucosidase) converts a considerable amount of the sucrose present into glucose and fructose, while the enzyme glucose oxidase converts small amounts of the glucose into glucono-lactone which is hydrolyzed to acid. The acid and hydrogen peroxide produced by the enzyme help preserve the honey (White, 1978). The bee carries the contents of its honey sac into the hive and regurgitates it in form of a droplet which is transferred to the house bee through contact with the antennae and front legs. The house bee then deposits the material into one of the cells in the hive. In the hive, the raw honey undergoes a "ripening" process in which the moisture content is decreased to about 20%. The bees sense when the honey has properly matured, fill the cells completely and seal them with an airtight wax capping (Crane, 1975).

The bee has several natural systems of protection from disease (Gojmerac, 1980). Worker bees instinctively remove foreign objects from the hive. Spaces or holes are filled or sealed with propolis, a sticky material gathered by the workers from buds and bark of trees. This prevents dirt and debris from accumulating in the hive. Old bees fly out of the hives to die while those that accidently die in the hive are immediately removed by the workers. In addition, methods of storing nectar and pollen in the hive help to maximize the safety of the colony. Pollen from a single source is stored in a single cell. This procedure prevents contamination of all the pollen crop if one batch is found to be toxic. The

modification of the nectar collected results in a raw honey product with low pH (3.2-4.5), low moisture content (< 20%) and high osmotic pressure which is also a means of defense against the growth of bacteria, fungi, and molds (Gojmerac, 1980).

Despite their instinctive defense methods, bees still suffer from several diseases. A common disease is American foulbrood, which is caused by *Bacillus larvae* and kills the larvae of the honey bees before they can develop into adult bees (Peng and Peng, 1979; Bailey, 1981; Shimanuki and Knox, 1988; Crane, 1990; Olsen et al., 1990). Another disease that affects the larvae is sac brood, which is a viral disease that prevents the larva from pupating normally (Gojmerac, 1980). The fungus *Ascosphaera apis* causes chalk brood, turning the larvae into mummies. This disease is characteristic of the cooler parts of the world, including Canada (Gochnauer and Hughes, 1976). Another fungus, *Aspergillus flavus*, causes stone brood, which is also fatal to the larvae. This disease occurs in hives with damp conditions and poor hive ventilation (Crane, 1990).

The most serious and most widespread disease of adult honey bees is the Nosema disease. It is caused by *Nosema apis*, a microsporidian protozoa (Gojmerac, 1980; Wilson and Nunamaker, 1983; Crane, 1990). Infection is caused by adult bees ingesting spores of the parasite, which develop within the epithelial cells of the gut of the adult honey bee. Worker bees normally fly out of the hive to void feces, reducing the possibility of contaminating others. But when workers are confined to the hive, because of cold weather, or when shipped in a package, the comb or inside of the package is soiled and the infection spreads to others. Nosema infection doesn't kill honey bees, but it shortens their lifetime. Queens are superceded much earlier and honey production decreases (Gojmerac, 1980).

Prevention and cure of Nosema disease is a major concern to honey producers. Nosema spores remain viable for up to 6 years when frozen, but are killed in 10 minutes in water at 57 °C. At 21 °C, spores remain viable for 2 months, but are killed when incubated at 35 °C in 21 days (Gojmerac, 1980). Therefore, live steam can be used to clean contaminated equipment and cages, while the combs can be sterilized by controlled heat

treatment. Fumigation with ethylene oxide or acetic acid is widely used not only to control Nosema, but also American foulbrood and wax moth infestation (Crane, 1990). In 1953, a new antibiotic, fumagillin, was first used as a means of protection against Nosema disease, and proved to be very effective when used as part of a good management system (Katznelson and Jamieson, 1952). However, honey producers are now concerned about the possible contamination of the honey by the antibiotic and, therefore, studies are needed to investigate this possibility.

The honey produced by the bee is marketed in different forms. It is either sold in the comb, as comb honey, to consumers who prefer it in its natural state, or the raw honey is processed, and sold as creamed or liquid honey. To produce the creamed product, pasteurized liquid honey is thoroughly mixed with starter crystals (glucose) and stored at a constant temperature of 13 °C. It is usually firm in three days and ready for market in about 6 days (Crane, 1975). The most widely accepted honey product is liquid honey. This is produced by heating to pasteurize and dissolve any crystals present, followed by filtration and packaging. Special care is taken, both in processing and storage, to avoid recrystallization of the product. Consumers prefer the liquid form because of its clear, shiny appearance and ease of handling. However, sometimes, problems are encountered by the producers and retail merchants when crystallization does occur in the liquid product. This is often the case with honey produced in Canada. Therefore, it is important to investigate the specific conditions and factors that may delay crystallization in honey.

This research deals with two main aspects of quality control in honey. The first part aims at determining the main factors that lead to crystallization of raw and processed liquid honeys produced in the province of Alberta, a major Canadian honey producing province, and methods to delay the process as much as possible. The other focus of this research is the development of accurate and precise analytical methods to detect the presence, if any, of fumagillin in honey.

A. CRYSTALLIZATION OF HONEY.

Crystallization of honey, commonly called granulation, is a physical phenomena. It may occur in the honey at any stage: production, processing or distribution to the consumer. This process is particularly undesirable in liquid honey due to several factors. Crystallization causes the product to become cloudy and, therefore, less appealing to the consumer. In addition, honey may contain yeasts (of the genus *Zygosaccharomyces*) which grow only in concentrated sugar solutions (Gojmerac, 1980). When crystallization occurs in the honey, the liquid phase has a higher water content than the entire honey had when it was all liquid. This high moisture content allows yeast spores that may be present to grow, which results in fermentation of the product (Doner, 1977).

The main components of honey are glucose, fructose, sucrose and water. Glucose crystallizes from solution in concentrations ranging between 30 and 70% depending on temperature. Fructose crystallizes only if present in concentrations of 78-95% (Gojmerac, 1980). Since most honeys contain about 30-40% glucose and 35-45% fructose (Table 1, adapted from Crane, 1990), they are supersaturated with respect to glucose. As a result, glucose in honey tends to crystallize spontaneously at room temperature in the form of glucose monohydrate (White, 1978). This tendency is enhanced if nuclei are present in the honey. These can be glucose hydrate crystals already present (or deliberately added, as in the production of creamed honey), air bubbles, pollen grains, or any other particulate matter. Even particles floating in the air act as nuclei and cause honey to crystallize. In addition, during the process of extraction of honey from combs, the honey is subjected to much agitation and this increases the rate of subsequent crystallization.

The speed with which glucose crystals form in honey is affected by changes in temperature, which in turn affects the viscosity. Honey is "thinner" when warm and "thicker" when cool. Glucose crystals are unable to grow unless molecular movement can occur in honey. When temperature is decreased, supersaturation of glucose in honey is increased, favouring crystal growth. However, the lower temperature also increases

viscosity and this reduces the diffusion of the glucose molecules, retarding the formation of crystals. There is a critical temperature for each honey at which the effect of supersaturation and viscosity balance out, and at this temperature, crystallization progresses most rapidly. For most honeys, this temperature is about 14 °C. Honeys with low moisture content crystallize faster at 15 °C. Crystallization is greatly retarded at temperatures less than 10 °C, while no crystal growth occurs at temperatures less than 4.5 °C. Even samples to which fine crystals have been added as nucleators showed no crystallization for two years when stored at temperatures less than 4.5 °C (Crane, 1975).

Table 1. AVERAGE COMPOSITION OF HONEY PRODUCED IN FOUR COUNTRIES.

Component	USSR	USA	Romania	Australia	Average	Range
Water	18.6	17.2	16.5	15.6	17.0	13.4 - 26.6
Fructose	37.4	38.2	38.4	43.3	39.3	21.7 - 53.9
Glucose	35.9	31.3	34.0	30.2	32.9	20.4 - 44.4
Sucrose	2.1	1.3	3.1	2.5	2.3	0.0 - 7.6

Since crystallization of liquid honey is highly detrimental to the honey industry, several attempts have been made to precisely predict the tendency of a honey to crystallize and determine effective ways of delaying this process (Crane, 1975; White, 1978). Attempts to understand honey crystallization using model systems of carbohydrates have been made (Doner 1977; White, 1978). Solubility curves of solutions exhibit three regions: the stable (unsaturated) zone where no crystallization occurs, the metastable

(supersaturated) zone where spontaneous crystallization occurs only in the presence of nucleators, and the unstable (supersaturated) zone where spontaneous crystallization is possible but not inevitable. Many factors affect the width of the metastable zone, and hence the rate of crystallization. The condition of supersaturation alone is not sufficient for a system to begin crystallization. The thermal history of the solution and the presence of nuclei are among the important factors that determine crystallization in a solution (Mullin, 1972). The presence of fructose also modifies the saturation point of glucose. Studies on the solubility of glucose in solutions of fructose, approximating those found in honey, indicate that the solubility of glucose increases with increasing fructose concentrations (White, 1978). This was explained by the existence of an equilibrium between anhydrous glucose and glucose monohydrate (the form found in crystallized honey). At high fructose levels, the equilibrium favors the anhydrous form, which shows a greater solubility in water. An abrupt increase in glucose solubility occurs in glucose-fructose-water systems at a fructose concentration of 150 g/100 g water. This may be related to the extent of hydration of the glucose in solution. Kelly (1954; 1955) observed that the transition temperature from glucose monohydrate to anhydrous glucose, in solutions saturated with fructose, is below 30 °C. In glucose-water systems, β-D-glucose monohydrate is metastable at 38 - 50 °C, but transforms to stable α-D-glucose monohydrate at 32 - 38 °C.

Generally, more glucose and lower moisture content increase the tendency of a honey to crystallize. Previous researchers have attempted to predict the tendency of a honey to crystallize using ratios involving the composition of honey with respect to glucose. A honey with glucose/water ratio < 1.7 tends to remain liquid for a long time, while one with a ratio > 2.1 usually crystallizes within weeks (Doner, 1977). In addition, since the presence of fructose affects the saturation point of glucose, other ratios, like fructose/glucose and (glucose - water)/fructose, were proposed to include the effect of fructose composition (Dyce, 1931). However, the use of these ratios to predict

crystallization is possible only when the honey samples examined differ in carbohydrate composition.

Several steps are taken, during processing, to prolong the liquid state of the honey. Pasteurization delays the process of crystallization, by dissolving any crystals that may be present in the crude product, but does not inhibit it completely. Pasteurization also kills yeast cells and, thereby, eliminates the possibility of fermentation of the honey. Yeasts are destroyed by heating at 63 °C for 7.5 min, 69 °C for 1 min or 71 °C instantaneously. Overheating of honey is not recommended since it results in formation of excessive amounts of hydroxymethylfurfural (HMF), through acid-catalyzed dehydration of hexose carbohydrates, especially fructose. The formation of HMF is usually accompanied by darkening and loss of flavor. Therefore, as little heat is applied to honey for as short a time as possible (Crane, 1975).

Filtering the processed liquid product under pressure gives the honey a clear brilliant color and removes the potential nuclei for crystallization such as undissolved glucose crystals, air bubbles, pollen grains or any other particles (Crane, 1975). The use of ultrasonic waves (9 kilocycles/second, 15 - 30 minutes) has also been reported to lead to destruction of yeast cells and delay crystallization for 15 months (Kaloyereas and Oertel, 1958).

Cooling honey is known to be a fairly efficient way to delay its crystallization. Some researchers recommend heating honey to 77 °C (171 °F) for 5 minutes, cooling rapidly to room temperature, bottling and storing the bottled honey at 0 °C (Crane 1975). Honey stored at 0 °C for at least 5 weeks, and subsequently stored at room temperature, remains liquid for over two years whereas samples of the same honey stored at 14 °C without previous storage at 0 °C crystallize within weeks. Precooling of the bottles of honey prior to placing them in cartons also helps prolong their liquid state (Crane, 1975). Honey frozen rapidly to - 45 °C or colder and then stored at such temperatures will keep indefinitely without change for long periods (Kaloyereas and Oertel, 1958). However,

cooling honey is not favorable commercially, since it is more economical to store the honey at room temperature.

Crystallization can be inhibited by the use of chemicals. Addition of 0.3% isobutyric acid solution to honey prevented crystallization for at least a year. When bees were fed 0.25 % and 5% of the acid in sugar syrup, the resulting honey was clear. Sorbic acid can also be used to inhibit growth of microrganisms in honey (Kaloyereas and Oertel, 1958). However, the use of chemicals in honey production is regarded as highly unfavorable and is restricted by regulating agencies.

In Canada, the province of Alberta is a major honey producing area, but most Alberta honeys are naturally granular. The final processed liquid honey appears to crystallize much faster than honeys produced in other areas. This may be partly due to the floral sources available and their carbohydrate composition, or the low relative humidity in the area. It may also be due to certain factors that may occur during processing. Since there are no definite recommendations available to the Canadian processor to ensure prolonged shelf-life of the liquid product, this study examines the honey at each stage of its production to determine which factors can be adjusted or modified in order to ensure a liquid product that does not crystallize for as long a period as possible.

B. ANALYSIS OF CARBOHYDRATES IN HONEY.

Carbohydrates can be identified and/or quantitated using different methods (Olechno et al., 1987; Lord et al., 1988; Pukl and Prosek, 1990). They have been identified using paper electrophoresis, reaction with spray reagents, and by chemical reactions like hydrolysis of higher saccharides to lower saccharides (Doner, 1977). However, the most common techniques are enzymatic and chromatographic.

Enzymatic analyses are highly sensitive and specific. They have been used for analysis of several carbohydrates, especially monosaccharides (Tzouwara-Karayanni and Crouch, 1990). Glucose can be identified using the enzyme glucose oxidase (Gojmerac,

1980). Commercial glucose oxidase (with contaminating α-glucosidase activity inhibited by Tris buffer) catalyzes the specific oxidation of glucose to gluconic acid. The hydrogen peroxide produced as a byproduct reflects the glucose content and is determined photometrically in an assay procedure using peroxidase. Although useful in most cases, enzymatic methods of analyses have several disadvantages. They are limited by interferences from contaminants in the test mixture (including other carbohydrates), source and purity of the enzyme used and the need for different sets of enzymes and conditions of analyses for each type of carbohydrate (Olechno et al., 1987).

Chromatographic techniques are the most widely used for determination of carbohydrates (Olechno et al., 1987). Paper and thin layer chromatography are used for identification of some carbohydrates, but have very limited use in quantitation. Gas and high performance liquid chromatographic methods are the most useful. Gas chromatography (GC) has been used for analysis of carbohydrates in honey (Folkes, 1985; Mateo et al., 1987). Mateo et al. (1987) converted the carbohydrates in honey into their trimethylsilyl ethers and oxime trimethylsilyl ethers and separated each set of derivatives by gas chromatography on a fused silica capillary column. Comparison of the results of the two chromatograms was used for identification of the carbohydrates present. GC, although a highly sensitive method, also has several disadvantages. It is necessary to prepare derivatives of the carbohydrates in order to render the carbohydrate volatile. If incomplete derivatization occurs, multiple peaks appear on the chromatogram. In addition, GC is a destructive method when used for carbohydrate determination, and cannot be used for preparative work. On the other hand, high performance liquid chromatography (HPLC) is an easier and faster method (Folkes and Crane, 1988; Ball, 1990; Lee, 1990; Swallow and Low, 1990).

Different HPLC columns are available for carbohydrate analysis (Rajakyla and Paloposki, 1983; Honda, 1984; Pirisino, 1984). The most common ones are the aminopropylsilica columns (Folkes and Crane, 1988). The various sample molecules are

separated by hydrogen-bonding interaction of hydroxyl groups on the carbohydrates with the polar amino group of the aminopropylsilica phase. These columns are relatively easy to use and have been used for analysis of several food products (Hunt et al., 1977; De Vries et al., 1979; Hurst et al., 1979; Damon and Pettitt, 1980; Nikolov et al., 1985), including honey (Thean and Funderburk, 1977; AOAC, 1990a). However, their use is, at present, not recommended (Brons and Olieman, 1983) due to their extensive interaction with the carbohydrates to form Schiff bases. They also require the use of acetonitrile as eluent, which is both expensive and toxic. In addition, although disaccharides are well separated on the aminopropyl columns, the analysis time is relatively long when compared to that of other columns.

Another type of silica-based column used for carbohydrate analysis is the octadecylsilyl reversed-phase columns in which the packing material is silica bonded to an 18-carbon alkyl chain. These are more efficient in separating higher oligosaccharides than monosaccharides such as glucose, fructose and sucrose (Cheetham et al., 1981; Rajakyla, 1986; Wight and Datel, 1986).

An HPLC system for carbohydrate analysis was recently introduced by Dionex (Townsend et al., 1988; Dionex, 1989; Garleb et al., 1989). It utilizes polymeric non-porous pellicular beads and pulsed amperometric detection. Resolution of the carbohydrates is achieved by anion-exchange mechanisms relying on the fact that at high pH (12-14), carbohydrates are either partially or completely ionized. Although the Dionex system is reported to be very efficient, its use requires the purchase of a system designed to withstand the highly basic conditions of operation. Since the non-metallic system is quite expensive, it is not yet available in many laboratories.

Cation-exchange resin columns are the most useful columns available for carbohydrate analysis (Schmuckler, 1987). They are generally prepared from sulfonated polystyrene crosslinked with divinylbenzene containing Ca^{++}, Ag^{+}, Pb^{+}, or H^{+} cations. Different cations form different complexes with the hydroxyl groups on the carbohydrates.

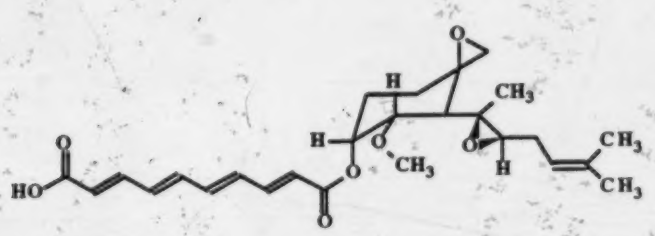
The position of the hydroxyl group in space (axial or equatorial) and the ability of the cation to form complexes determine the retention and selectivity obtained during analysis (Goulding, 1975; Briggs et al., 1981). Size exclusion and hydrophobic adsorption mechanisms are also involved in the process (Parris, 1984). Generally, the cation-exchange resin-based columns have a longer life-time than silica columns, with enhanced selectivity. The eluent needed in most cases is water, which is inexpensive and non-toxic, and the resins have reasonable mechanical stability. In addition, the time needed for analysis on cation-exchange resin columns is almost half that needed for similar analyses on silica columns. Cation-exchange resin columns are, therefore, the most suitable columns for analysis of sucrose, glucose and fructose in honey.

C. FUMAGILLIN.

Fumagillin (Figure 1) is an antibiotic obtained from *Aspergillus fumigatus* (Eble and Hanson, 1951; Girolami, 1963; Wilson, 1971). Several chemical reactions have been performed on fumagillin, especially by Tarbell and co-workers, in an attempt to determine the molecular structure of the compound (Schenck et al., 1955; Tarbell et al., 1955; Landquist, 1956; Ross et al., 1956; Chapman and Tarbell, 1958; Cross and Tarbell, 1958; McNally and Tarbell, 1958; Tarbell et al., 1961). It is a polyene antibiotic, with a tetraene chain conjugated to a carboxylic acid group (Schenck et al., 1953; Tarbell et al. 1960; Turner and Tarbell, 1962).

The configuration of the molecule was studied by x-ray crystallography as the mono-*p*-bromo benzene sulfonate derivative (Wilson, 1971). Studies on the biosynthesis of fumagillin, using acetic acid- ^{14}C, indicate that the polyene side chain is obtained from acetate, the terminal carboxyl arising entirely from the carboxyl in acetate, while the rest of the molecule is produced by a terpenoid route (Birch and Hussain, 1969; Wilson, 1971). A total synthesis of (±) fumagillin has been reported by Corey and Snider (1972).

Figure 1 FUMAGILLIN



Fumagillin has limited antibacterial or antifungal activity and little or no effect against several common animal viruses, but it is of interest mainly because of its amebicidal action. Fumagillin is reported to inhibit RNA synthesis in microsporidian pathogens (Jaronski, 1972). It shows activity against at least 7 enteric protozoan parasites, being most effective against *Entamoeba histolytica*, and has been used as an antibiotic in adolescent and adult male patients who were hospitalized because of infection with *Entamoeba histolytica* (Killough et al., 1952; McCowen et al. 1951). However, serious side effects were observed from the therapeutic doses of fumagillin in man (Wilson, 1971). Therefore, attempts have been made to synthesize analogues of fumagillin (Harvey and Miners, 1979). Recently, Ingber et al. (1990) reported the effectiveness of fumagillin in suppressing tumour growth, but severe weight loss was observed in the test animals during the treatment of the tumour, which prompted Ingber and co-workers to synthesize several analogues of fumagillin. The replacement of the long polyene chain (R-O-CO-$(C=C)_4$-COOH) with a chloroacetylcarbamoyl moeity (R-O-CO-NH-CO-CH_2Cl) gave a compound that was highly efficient in suppressing tumour growth and no weight loss was observed in the test animals. Fumagillin is also effective in the treatment of a number of parasites in fish (Lauren et al., 1989).

In the honey industry, fumagillin is of interest due to its effectiveness in protecting bees against *Nosema apis* (Katznelson and Jamieson, 1952; Woyke, 1984). It does not kill spores, but protects worker bees against infection when it is fed to them. Approximately 5 grams is suspended in 3750 mL of sugar syrup and fed to the colony in the fall after brood rearing is reduced or stopped (Gojmerac, 1980).

Fumagillin has an LD_{50} of approximately 800 mg per Kg body weight by subcutaneous administration in mice. Orally in mice, fumagillin was tolerated at doses up to 2000 mg per Kg body weight (Eble and Hanson, 1951). Toxicological studies were performed by Chinoin Toxicological Laboratories (Blasko and Englovski, 1990) using a 1% methylcellulose suspension of fumagillin dicyclohexylamine salt (FDCH, the commercially available form of the antibiotic) as oral treatment. Table 2 shows the LD_{50} obtained for male and female rats and rabbits.

Table 2. LD_{50} OF FUMAGILLIN DICYCLOHEXYLAMINE SALT (mg/Kg BODY WEIGHT) IN ORAL TREATMENTS AS A 1% METHYLCELLULOSE SUSPENSION.

ANIMAL	MALE	FEMALE
Rat	3474	2759
Rabbit	1447	2000

The toxicity of FDCH for the honey bee itself has also been determined (Hadhazy and Zajak, 1986). FDCH was diluted in a sugar syrup in different concentrations and fed to the bees. The LD_{50} was found to be 512.0 µg/bee. Since an $LD_{50} > 100$ µg/bee is regarded

as non-toxic in bee toxicological practises, fumagillin does not seem to present any hazard to the bees when it is fed to them in the usual doses.

However, since there is a lot of concern about the use of chemicals in honey production, it is important to determine the fate of fumagillin once it has been ingested by the bees and whether or not it gets into the honey.

In addition, fumagillin is known to be unstable in light (Garrett and Eble, 1954; Eble and Garrett, 1954) and heat (Garrett, 1954). Fumagillin, if not stored in dark bottles in the absence of oxygen and at low temperature, looses its activity against *Entamoeba histolytica* and *Staphylococcus aureus* phage. Concurrent with this activity loss is a loss in UV absorption over a period of months (Schenck et al., 1955).

Fumagillin possesses a strong chromophore in the ultraviolet with absorbance maxima at 336 and 351 nm which can be attributed to the conjugated acid tetraene portion of the molecule. Preliminary stability studies have shown correlation between loss in biological activity and loss in absorbance of these chromophores when fumagillin was exposed to attack of air, light or heat; both in and out of solution. In order to determine whether the cause of degradation of fumagillin in solution when exposed to light was photolytic, photooxidative or oxidative, Garret and Eble (1954) performed an experiment where tubes of fumagillin in ethanol were placed under a light source. Oxygen was bubbled into covered and uncovered tubes, and nitrogen was bubbled into covered and uncovered tubes. After 6 hours the fumagillin content of each tube was assayed by the *S. aureus* phage technique. It was observed that decomposition occurred in tubes uncovered from light irrespective of presence or absence of oxygen. Thus, the destruction of fumagillin under light is primarily photolytic. In another experiment, it was found that fumagillin in ethanol solution is most readily destroyed by wavelengths of light below 400 nm. None of the products of degradation of fumagillin in ethanol significantly absorb at wavelengths greater than 345 nm. Garrett and Eble (1954) suggested that the fumagillin chromophore is transformed to new chromophores (termed neofumagillin) which absorb at lower

wavelengths. The neofumagillin(s) in ethanol solution are also photolytically degraded at pseudo-first order rates but more slowly than fumagillin.

Crystalline fumagillin exposed to light and air for one year lost 90 % of its absorptivity at 351 nm. Absorbances at wavelengths below 300 nm increase as the fumagillin chromophore is destroyed, but no new chromophore was recognized. Photolysis of crystalline fumagillin in the presence of air is accompanied by oxidation and an increase in acetyl content, most probably in the alcohol portion of the molecule (Eble and Garrett, 1954).

Garrett (1954) also studied the thermal degradation of crystalline fumagillin in the presence and absence of air. He observed that the tetraenedioic chromophore is thermally destroyed in the presence and absence of air by different mechanisms. In the presence of air, it appears that the degradation occurs by reaction of one molecule of oxygen with two molecules of fumagillin. Complete transformation to several chromophores which absorb at a lower wavelength occurs. Degradation under nitrogen is pseudo zero order initially to about 80 % of the absorptivity of pure fumagillin. Further chromophore degradation is not linear with time and the extent is a function of temperature (Garrett, 1954)

Garrett (1954) also observed that the fumagillin chromophore is relatively stable to heat in ethanol under nitrogen. After an initial thermal degradation, fumagillin in ethanol solution maintained 80 % of its absorptivity even at 70 ^{o}C for 35 days.

Since there is no precise information available on the mechanism of decomposition of fumagillin or the identity of the products formed, a study of the photolytic and thermal degradation of the antibiotic and the resulting effect on the honey is important in determining the fate of the compound during the process of honey production.

D. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

Immunoassays are highly sensitive and specific techniques, and are now widely used for detection of residues in food (Morris and Clifford, 1985; Tijssen, 1985; Dixon et

al. 1986; Gosling, 1990). In immunoassays, the compound to be detected (the antigen) is injected into an animal, usually a rabbit. Antibodies (large Y-shaped proteins) are developed in the immunized animal specifically against the injected antigen (Coleman et al. 1989; Paul, 1989). Substances with a molecular weight less than 5000 dalton are not ordinarily antigenic. However, antibodies can be raised to these small non-immunogenic molecules (known as haptens) by immunization with conjugates made up of the low molecular weight substances covalently linked to proteins or synthetic polypeptides. The high molecular weight polypeptides or proteins have a large number of different amino acid residues which create a larger diversity in epitopes (sites of attachment to antibodies), resulting in stronger immunological response. The blood from the immunized animal, containing a high concentration of the required antibodies, is collected from the animal and the blood cells are allowed to coagulate. The resulting liquid portion, the serum, now contains the antibodies required for the immunoassay.

The specific high affinity, reversible binding of the antibodies to the specific antigens they were raised against results in the high levels of sensitivity and specificity achieved in immunoassays (Tijssen, 1985). An immunoassay is generally based on the principle that the likelihood of a molecule of antigen to bind to the antibody is proportional to its concentration in the original antigen mixture (Butler, 1980). Either the antigen or the antibody is labelled and the label can either be a radioactive isotope (radio-immunoassays, RIA), a fluorescent label (fluoroimmunoassay), or an enzyme (enzyme-immunoassays, EIA).

RIA is an extremely sensitive, specific and accurate method of analysis (Dwenger, 1984; Dixon et al., 1986; Chard, 1987). It requires only small amounts of sample and very short analysis time. However, its disadvantages outweigh its advantages. The most commonly used isotopes in RIA (^{125}I and ^{131}I) have short half-lives which leads to increased costs for quality assurance and distribution. When incorporated into substances, the radioactive disintegration of the isotopes is destructive to molecular structure and special

precautions are required due to the health hazards involved. The high cost of the instrumentation needed for quantitation of radioactivity is also a drawback to the wide use of the technique (Clark and Engvall, 1980). As a result, a number of non-isotopic immunoassays have been developed to avoid the problems encountered when working with radioimmunoassays. In fluoroimmunoassays, fluorophore labels are used for haptens that possess fluorescence spectra distinctly different from those of the antibody (Hammila, 1985; Diamandis, 1988). Enzyme-immunoassays (EIA) use an enzyme as label and are fast becoming a major scientific and analytical tool (Oellerich, 1984; Tijssen, 1985).

EIA are rapid, simple assays with very high sensitivity, detectability and specificity. There are no radiation hazards and the equipment and reagents are relatively cheap. Also, reagents have long shelf lives. The assay has good reproducibility and many forms of the assay are feasible under field conditions. Moreover, the great variety and specificity of enzymes increases the versatility of the assays. EIA can also utilize monoclonal antibodies which are now regarded as an extremely useful tool in assays (Goding, 1986; Schook, 1987).

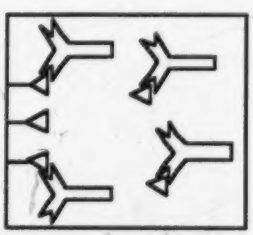
Enzyme-linked immunosorbent assay (ELISA) is an EIA that can be used to detect and determine concentrations of antigen or antibody. There are different techniques used in ELISA (Clark and Engvall, 1980). A common type of ELISA, the indirect competitive ELISA (Scheme 1), involves attaching the antigen to a solid phase, usually a plastic plate. A solution of standard or test antigen is then added and both bound and free antigen are allowed to compete for the antibodies in the serum. Antibodies that bind to the free antigen are washed off while antibodies that become immobilized on the plate through attachment to the bound antigen are allowed to bind to enzyme-labelled anti-antibodies. Enzymes used for labelling are usually horseradish peroxidase, β-galactosidase or alkaline phosphatase. They are all stable, relatively low priced, can be easily linked to the antibody or antigen and have a suitable chromogenic substrate. Horseradish peroxidase is the most widely used because it is less expensive than the other enzymes and produces easily detectable products, even

Scheme 1. INDIRECT COMPETITIVE ELISA.

1. Attach antigen to solid phase.

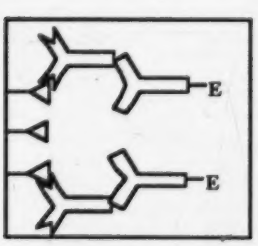
2. Wash

3. Incubate with antibody in presence of sample.



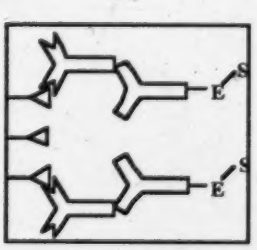
4. Wash.

5. Add enzyme-labelled secondary antibody.



6. Wash.

7. Add substrate and measure absorbance.



though determination of its activity involves several sensitive redox reactions and the substrate, H_2O_2, is unstable (Welinder, 1979; Clark and Engvall, 1980). The amount of solid phase-bound enzyme activity is quantitated by the enzyme-catalyzed conversion of the substrate into a highly chromatic product. The amount of color produced is proportional to the amount of labelled anti-antibodies bound to the plate and therefore related to the amount of hapten in the sample (Clark and Engvall, 1980).

Another type of ELISA is the direct competitive ELISA, where the antibodies are adsorbed to the bottom of the wells, then both the sample to be analyzed and the labelled hapten are added and allowed to compete for the immobilized antibody. The substrate for the enzyme is then added and the amount of color produced is measured. In the sandwich ELISA, the antibody is immobilized on the solid surface and test sample is added to form an immobilized immune complex. Addition of enzyme labelled antibody results in formation of an immobilized antibody-antigen-antibody complex. The substrate for the enzyme is then added and the intensity of the resulting color is proportional to the amount of free hapten in the sample.

ELISA techniques have some limitations. Immobilization of antigen or antibody is achieved by hydrophobic interactions between the nonpolar protein structure and the nonpolar plastic carrier. Since the antigen or antibody is physically and not covalently bound, some adsorbed protein is lost during washings, leading to a decrease in the precision and sensitivity of the assay. Interactions between antibodies and their antigens also do not involve covalent bonds. Rather, they include forces like van der Waals forces, electrostatic interactions, hydrogen bonds and hydrophobic interactions (Butler, 1980). The strength of antibody-antigen interactions vary with pH, presence of ions (iodide, bromide, chloride) and temperature. Presence of organic solvents may also disrupt the van der Waals bonds and dissociate the antibody-antigen complex. In addition, the quantity of antibody which can be attached to the wall of a well of a microtitre plate is limited by the surface of

the well and the fraction of antibodies present in the serum. However, the ease and speed of the ELISA technique compensates for these drawbacks (Clark and Engvall, 1980).

CHAPTER 2

CRYSTAL CONTROL IN PROCESSED LIQUID HONEY

Several factors may be investigated when attempting to determine methods of delaying crystallization in specific honeys. Different geographical areas with different floral sources may, in principle, yield honeys that crystallize at different rates. It would, therefore, be useful to identify producers, if any, that can provide raw material adequate for production of the liquid product. If this is not possible, then manipulations could be performed during the processing stage. These could be in the form of blending raw honeys to certain compositions of carbohydrates and moisture in an attempt to produce a product that would remain liquid for a longer period. In addition, since temperatures affect crystallization, a study of the thermal history of the different honey samples may be an important approach to determining the main factors involved in this complex process.

A. ORIGIN OF THE RAW MATERIAL.

Different geographical areas with different floral sources may yield honeys that crystallize at different rates. It would, therefore, be useful to identify producers in the province of Alberta that can provide raw material to be used for the processed liquid honey product on a regular basis.

Differences between crude products could be due to either the floral source, composition of the nectar or environmental conditions. The floral source may depend on the geographical location of the sample. The main honey producing areas in Alberta are: Peace River area in the north, central Alberta around the Provincial capital, Edmonton, and southern Alberta. The main floral sources in these regions are clover, canola (rapeseed), and alfalfa. Unprocessed canola honey has negligible amounts of sucrose and is known to

crystallize naturally, while alfalfa contains sucrose and has high glucose and fructose content (Table 3; adapted from Low et al., 1988). In contrast, clover honey has low amounts of glucose and fructose and remains liquid for a longer period. Therefore, knowledge of the clover content of the honey could be useful in predicting its crystallization properties.

Table 3. PERCENTAGE SUGARS IN NECTAR FROM FLORAL SOURCES AVAILABLE IN ALBERTA, CANADA.

Floral Source	% Fructose	% Glucose	% Sucrose
alfalfa	17	12	5
canola	9	10	none
sweet clover	9	7	7

Raw honey samples from different parts of the province were examined to determine if their geographical origin and/or clover content could indicate a specific honey source ideal for production of liquid honey. Information on the clover content of these samples (Table 4) was provided by the producing company, BeeMaid Ltd, where the clover content was determined by dissolving a sample of the honey in hot water, centrifuging and examining the sedimented pollen on a microscope. At least 50 pollen grains are identified and the percentage of the grains found to be clover reported.

Clover analysis is routinely performed on all crude honey samples before processing as a means of identifying its floral sources. This information would only be useful in prediction of crystallization if it correlates with differences in composition. Although, generally, higher clover content indicates lower % glucose and, therefore, a

Table 4. GEOGRAPHICAL ORIGIN, CLOVER CONTENT AND APPEARANCE OF RAW ALBERTA HONEY SAMPLES.

Sample No.	Geographical Origin	% Clover	Appearance[1]
A - PEACE RIVER SAMPLES			
1	Peace River	50	liquid
2	Falher	84	solid
3	Grande Prairie	83	liquid
4	Peace River	59	solid
5	High Prairie	91	semi-solid
6	Valley View	85	liquid
7	Falher	63	solid
8	Girouxville	90	solid
	Average % clover for Peace River samples	**76**	
B - CENTRAL ALBERTA SAMPLES			
9	Drayton Valley	32	liquid
10	Barrhead	43	solid
11	Drayton Valley	97	liquid
12	Toefield	53	solid
13	Alsike	96	solid
14	Smokey Lake	4	solid
15	St. Paul	3	solid
16	Drayton Valley	88	solid
17	Mayerthorpe	63	solid
18	Mayerthorpe	31	solid

19	St. Paul	7	solid
20	Wetaskiwin	59	liquid
21	Wetaskiwin	36	solid
22	Barrhead	52	semi-solid
23	Westlock	87	solid
24	Barrhead	60	semisolid
25	Wildwood	10	solid
26	Edmonton	62	solid
27	Sangudo	96	solid
	Average % clover for Central Alberta samples	52	

C - SOUTHERN ALBERTA SAMPLES

28	Medicine Hat	50	solid
29	Olds	68	liquid
30	Scandia	50	solid
31	Cremona	4	solid
32	Medicine Hat	50	solid
33	Irricana	9	solid
34	Medicine Hat	25	solid
35	Olds	58	solid
36	Olds	30	solid
37	Medicine Hat	15	solid
38	Irricana	10	solid
39	Cremona	65	solid
40	Irricana	78	solid
41	Calgary	37	solid

Average % clover for Southern Alberta samples	**39**

1. Appearance after 2 years storage at 25 °C: liquid = no crystallization; semisolid = layers of crystals visible; solid = totally crystalline.

lower tendency of crystallization, this is not the case here, as seen from the results in Table 4. This may be due to the differences in moisture levels in the samples and possibly other factors like the size of the sample or the extent to which it was handled. It does appear that, on the average, raw honeys from the Peace River area are more likely to have a higher clover content and remain liquid for a longer period than honeys from other parts of the Province. Nevertheless, almost all the samples were crystallized with very few exceptions and, therefore, the clover content of the raw material and its geographical origin is not a sufficient criteria for controlling crystallization in Alberta honeys.

B. CARBOHYDRATE COMPOSITION OF RAW AND PROCESSED HONEY.

Differences in carbohydrate composition would affect the degree of supersaturation of glucose in the honey and, therefore, its crystallization properties. It is known that nectar with high glucose content crystallizes easily. For example, ivy nectar, which has an unusually high glucose content, crystallizes while still in the honey-stomach of the honey bee (Greenway et al. 1978). Residues of 70% glucose and only 22% fructose were found in the honey stomach. Also, honeys with glucose content less than 30% (e.g. tupelo, acacia) rarely ever crystallize while those that have about 37% glucose (e.g. cotton) are naturally crystalline (Crane, 1975). Therefore, analysis of glucose, fructose and water content may indicate the tendency of the honey to crystallize.

HPLC is a fast and easy method of analysis for carbohydrates. It does not require prior derivatization, as in GC, and samples can be prepared and analyzed in less than an hour. Thus, HPLC was our method of choice for analysis of the raw and processed honey samples.

HPLC analysis of carbohydrates in honey.

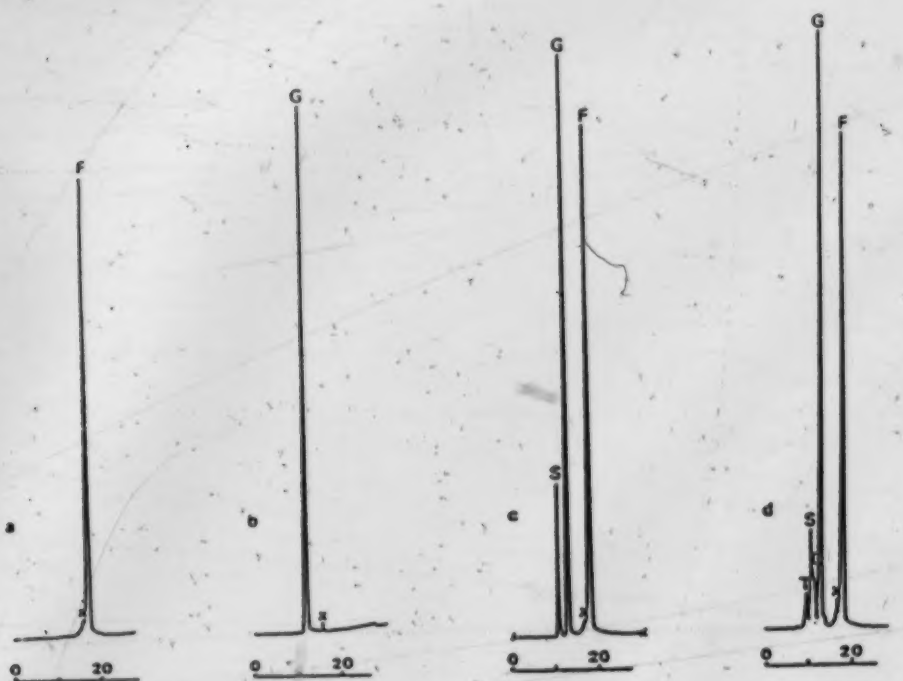
Cation-exchange resin columns are the most useful HPLC columns available for carbohydrate analysis (Schmuckler, 1987). The resin column loaded with Ca^{++} ion has

been extensively studied in the literature (e.g. Duarte-Coelho et al., 1985; Picha, 1985). It is especially useful in following lactose hydrolysis to glucose and galactose during the production of lactose-hydrolyzed milk (Brons and Olieman, 1983; Pirisino, 1983; Pirisino, 1984). Since the use of Ca^{++} columns has been fully developed, we decided to investigate the use of other cation-exchange resin columns.

The Aminex HPX-87P (Pb^{++}), from BioRad, can be used for separation of carbohydrates (Wentz et al., 1982; Vidal-Valverde et al., 1984; Bouchard et al. 1988). In an attempt to use this column for our analyses, we found it quite problematic. The column is operated at 85 °C using a column heater. This increases the possibility of formation of air bubbles on the column leading to distortion of the peaks and problems with reproducibility. Therefore, to prevent bubble formation, it was found necessary to keep degassed eluent, water, stirring at about 60 °C throughout the analysis. In addition, the lead column causes slight decomposition of the carbohydrates, as indicated by appearance of an extra peak on the chromatogram for both honey and standard carbohydrate samples (Figure 2). This verifies a report by Baker and co-workers (1988) that the ion exchange resin in the lead form causes degradation of fructose and glucose. They had observed that samples of monosaccharides analyzed on the lead column (60 °C in deionized water at a flow rate of 0.3 mL/min) produced extra peaks on the chromatogram which are not observed when the analysis is performed on a calcium column. When the products of the analysis on the lead column were run again on a calcium column, the extra peaks did appear, indicating that these peaks were not a result of better resolution on the lead column, but resulted from decomposition of the sugars during analysis.

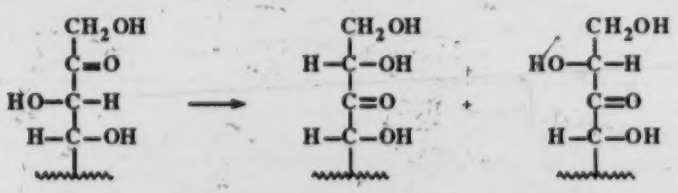
The degradation of carbohydrates by resins loaded with lead ions appears to be general with respect to 3, 5 and 6-carbon ketoses. Monosaccharides undergo a variety of degradation reactions when subjected to strong alkaline solutions. Although the analysis is performed in water, and, therefore, would be in a neutral medium, the lead ion has a very

Figure 2. **LEAD COLUMN CHROMATOGRAMS: a) Fructose standard; b) Glucose standard; c) Standard solution containing sucrose, glucose and fructose; d) Honey sample. Scale denotes retention time in minutes. F=fructose, G=glucose, S=sucrose containing peak, D=other dissacharides, T=trisaccharides, X=decomposition product.**



strong tendency to extract hydroxyl ions from water. Therefore, Baker and co-workers (1988) postulated that it was possible that sugar molecules complexed to lead ions were effectively in a highly alkaline environment due to the hydroxyl ions bound to the same lead ion. This would not occur in the case of the calcium ion since calcium's tendency to extract hydroxyl ions from water is about five orders of magnitude less than that of lead. The lead ion also has a strong tendency to accept coordinate bonds from oxygen. This would facilitate isomerizations involving the initial shift of the carbonyl function from the 2-carbon to the 3-carbon. Scheme 2 shows the products proposed by Baker and co-workers (1988) for degradation of monosaccharides on exposure to resins loaded with lead ions.

Scheme 2. ISOMERIZATION OF MONOSACCHARIDES ON LEAD RESIN COLUMNS.



The extent of degradation on the lead column increases with the tendency of the sugars to exist in the open chain form, duration of exposure to the lead ions on the resin, and the temperature at which the column is operated. Baker observed that D-fructose was degraded to an extent of < 3% after 12 h exposure to the lead-loaded resin at 60 °C. D-Glucose was reported to be far more resistant to the degradation than fructose. Therefore, the extent of degradation of the sugars during our analysis at 85 °C in distilled deionized

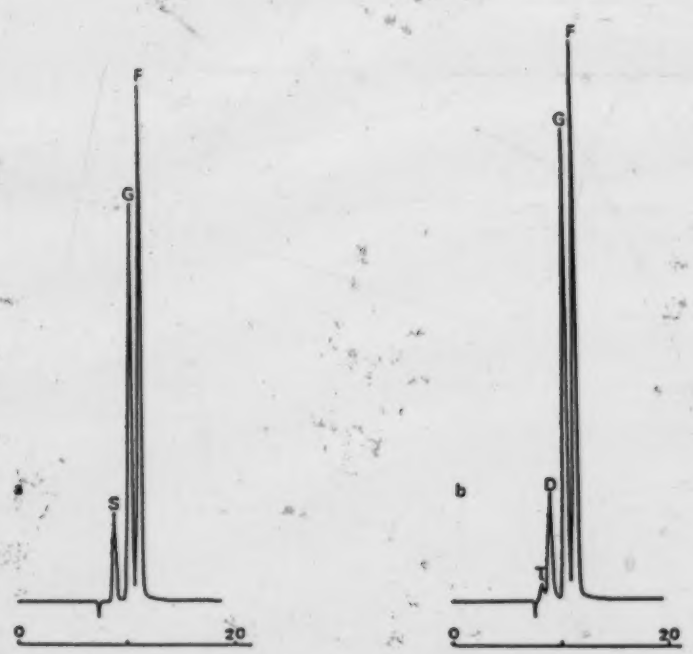
water at a flow rate of 0.6 mL/min (duration of the run was about 20 min) would have relatively little effect on the results, but an alternative column would still be preferable.

The use of a cation-exchange resin column in the H^+ form was investigated next. This column is normally used for analysis of fermentation products such as acids, and not carbohydrates (Pirisino, 1984), due to the fact that it is operated at elevated temperatures using dilute sulfuric acid as eluent. Under these conditions, sucrose is hydrolyzed to glucose and fructose. However, we established that, when the Rezex-H^+ column is used at room temperature, sucrose was not hydrolyzed and separation of glucose, fructose and sucrose was maintained, although there is slight broadening of the peaks. This column was found to be more reliable and easier to use than the lead column. The dilute sulphuric acid used as eluent served as a constant regenerator of the resin, thus reducing deterioration problems, and operation at room temperature eliminates problems encountered when a higher temperature was required. A typical chromatogram obtained for honey samples on the Rezex-H^+ column (room temperature, 0.01 N H_2SO_4) is shown in Figure 3.

Although both cation-exchange resin columns separated disaccharides from monosaccharides, there was not enough resolution of the individual dissacharides to accurately quantitate them. The lead cation-exchange resin column gave slightly better resolution of dissacharides, with a peak containing sucrose separated to some extent from other dissacharides (Figure 2), while all disaccharides coeluted on the acid cation-exchange resin column (Figure 3).

All analyses on the cation-exchange resin columns were performed in triplicate. The average coefficient of variance for all samples analyzed on the acid column was 0.26 % for fructose (about $\pm$ 0.1 % variation in determined values) and 0.34 % for glucose (slightly greater than $\pm$ 0.1 %). The greatest coefficient of variance in each case was 0.99 % and 1.51 % respectively. For the lead column, the average coefficient of variance for all samples was 1.09 % for fructose (about $\pm$ 0.4 %) and 1.11 % for glucose (also about

Figure 3. **ACID COLUMN CHROMATOGRAMS: a) Standard solution containing sucrose, glucose and fructose; b) Honey sample. Scale denotes retention time in minutes. F=fructose, G=glucose, S=sucrose, D=disaccharides, T=trisaccharides.**



± 0.4 %). The greatest coefficient of variance was 1.99 % for fructose and 2.92 % for glucose.

Composition of raw honey samples.

Table 5 shows results of analysis of crude honey samples obtained from the different producing areas in Alberta performed on either the Rezex-H^+ column at room temperature using 0.01N H_2SO_4 as eluent or the BioRad HPX-87P (Pb^{++}) column at 85 °C using distilled deionized water. The moisture contents were determined using an Abbe refractometer maintained at 20 °C using a constant temperature bath. The refractive index measurements were converted to moisture content using literature tables (Wedmore, 1955; Crane, 1975; AOAC, 1990b). The moisture content of most of the samples was determined after one year of storage at 25 °C, but some were two years old (marked with * in Table 5). The two year old samples had markedly lower moisture content than the other samples. This phenomenon of moisture loss is further discussed later.

Almost all the crude samples examined (Table 5) have high levels of glucose and low levels of water, which would explain the fact that they are almost all crystalline. The average glucose composition of all samples was 34.2 %, ranging between 30.9 - 39.9 %. Fructose values ranged from 35.4 - 41.7 % with an average of 38.6 % fructose. These sugar contents are high, but this may be due in part to the low moisture levels of these samples. The high sugar content is especially noticeable when compared to the range of composition of honeys reported in the literature (Table 1; Crane, 1975). The lowest value for glucose obtained in our Alberta samples was 30.9 %, while honeys in the U.S. may have glucose content as low as 22.0 %.

Table 5. COMPOSITION OF RAW ALBERTA PRODUCER HONEY SAMPLES.

Sample No.[1]	Column[2]	Glucose %	Fructose %	"Sucrose"[3] %	Water[4] %	Fructose/ Glucose
1	H	33.3	37.8	9.5	17.5	1.14
2	H	34.7	37.8	4.7	16.9	1.09
3	H	31.7	37.3	10.6	16.6	1.18
4	H	33.6	38.3	6.7	17.7	1.14
5	H	33.2	39.1	7.4	17.4	1.18
6	Pb	33.2	37.5	9.1	15.2	1.13
7	Pb	36.2	40.8	4.9	14.9	1.13
8	Pb	32.9	37.3	1.4	16.0	1.13
9	H	31.4	36.1	7.0	16.5	1.15
10	H	34.6	39.3	7.3	16.9	1.14
11	H	32.4	40.2	8.5	17.3	1.24
12	H	34.3	38.2	9.2	16.5	1.11
13	H	30.9	37.4	9.2	16.2	1.21
14	H	35.6	39.5	7.5	16.9	1.11
15	H	31.7	35.4	8.1	16.2	1.12
16	H	32.9	39.5	8.5	16.6	1.20
17	H	32.6	38.4	12.2	16.8	1.18
18	H	32.9	37.7	10.4	16.9	1.15
19	Pb	33.6	38.2	2.2	16.6	1.14
20	Pb	33.1	36.8	4.2	14.8	1.11
21	Pb	39.9	37.8	4.8	15.5	0.95
22	Pb	35.8	37.8	4.6	16.9	1.06

23	Pb	33.7	36.1	1.6	17.9	1.07
24	Pb	36.4	41.6	3.9	14.9	1.14
25	H	37.0	39.0	8.1	13.7*	1.05
26	H	36.2	38.6	8.4	14.6*	1.07
27	H	37.1	39.9	7.8	<13.0*	1.08
28	Pb	36.7	41.7	6.5	16.0	1.14
29	H	32.7	38.5	9.6	13.2*	1.18
30	H	32.7	37.4	10.9	13.5*	1.14
31	H	32.3	36.7	11.0	14.7*	1.13
32	H	34.7	39.3	10.6	<13.0*	1.13
33	H	35.0	39.2	9.5	13.2*	1.12
34	H	35.1	40.0	9.5	13.9*	1.14
35	H	33.6	38.9	10.1	13.6*	1.16
36	H	34.1	39.1	9.6	14.1*	1.15
37	H	36.3	39.8	7.0	13.5*	1.10
38	H	34.9	39.6	8.5	14.1*	1.13
39	H	33.5	39.1	9.6	14.2*	1.17
40	H	33.8	39.5	9.2	14.2*	1.17
41	H	35.8	38.4	6.7	13.2*	1.07
AVERAGE[5]		**34.2**	**38.6**			**1.13**
RANGE		**(30.9-39.9)**	**(35.4-41.7)**			

1. See Table 2 for geographical location and clover content of samples.

2. H = Rezex-H^+ column, 25 °C, 0.01 N H_2SO_4 at flow rate of 0.6 mL/min; Pb = BioRad HPX-87P (Pb^{++}) column, 85 °C, distilled deionized water at flow rate of 0.6 mL/min.

3. Sucrose on the Pb column was somewhat separated from other dissacharides (Figure 2), while sucrose on the acid column coeluted with the other dissacharides (Figure 3). Therefore, apparent sucrose ("sucrose") values obtained on the lead column were generally lower than those obtained on the acid column.

4. Water values with * indicate measurements were taken after two years of storage at 25 °C, all other values were obtained after one year of storage.

5. See Table 1 for comparison with values for other countries.

Looking at the fructose to glucose ratios, the variation in amounts of monosaccharides is remarkably small. Also, the sugar composition of honey samples that contained mostly clover pollen did not differ markedly from those with smaller clover content. This confirms the fact that no honey producing area in Alberta produces honey with low enough glucose content to be used exclusively as a raw material for the production of processed liquid honey.

Blending of raw honey.

In the processing plant, barrels of crude samples from the different honey producers are blended together in the "raw tank" and then processed to produce the final product. We, therefore, decided to determine if analysis of samples from individual barrels of crude honey can be used to predict the composition of the final blended product.

A sample was obtained from one of the barrels of each producer. These samples were analyzed for glucose and fructose using the BioRad HPX-87P (Pb^{++}) column. The average glucose and fructose content of the blended product was calculated, taking into consideration the number of barrels available from each producer. This calculated average was compared to the actual results obtained from analysis of a raw tank sample and three samples of the final processed honey (FH-1, FH-2, FH-3). The results (Table 6) indicate that this procedure could be used to predict the composition of the final product, although this prediction would not be precisely accurate due to sampling errors. To determine the extent of sampling error, samples from several drums from the same producer (i.e. containing the same crude honey) were analyzed. Table 7 shows that the coefficient of variance for samples from 5 drums was 1.76 % for fructose and 2.33 % for glucose. When one sample was analyzed several times, the coefficient of variance was found to be 0.55 % for fructose and 1.33 % for glucose.

Table 6. CARBOHYDRATE ANALYSIS OF CRUDE, RAW TANK AND FINAL HONEYS[1]

Ser. No.[2]	No. of barrels[3]	% Glucose (± s)[4]	% Fructose (± s)
1	4	33.2 ± 0.1	37.4 ± 0.1
2	12	33.6 ± 0.1	38.2 ± 0.9
3	16	33.1 ± 0.5	36.8 ± 0.1
4	1	36.7 ± 0.6	41.7 ± 0.2
5	2	39.9 ± 0.5	37.8 ± 0.6
6	1	35.8 ± 0.4	37.8 ± 0.4
7	1	33.7 ± 0.4	36.1 ± 0.4
8	1	36.2 ± 1.1	40.8 ± 0.6
9	1	36.4 ± 0.1	41.6 ± 0.8
10	1	32.9 ± 0.3	37.3 ± 0.7
CALCULATED AV. OF 40 BARRELS:		35.2 ± 2.1	38.6 ± 1.9
RAW TANK		34.6 ± 0.2	40.0 ± 0.8
FH-1		33.5 ± 0.6	37.7 ± 1.0
FH-2		33.4 ± 1.6	37.1 ± 0.5
FH-3		32.7 ± 0.1	38.3 ± 0.2

1. Column: HPX-87P (Pb^{++}), 85 °C, H_2O, 0.6 mL/min.

2. FH-1, FH-2, FH-3 are 3 samples of final product after processing.

3. Number of barrels of the sample used in the raw tank.

4. Values are % sugar ± standard deviation of triplicate analyses, except for the calculated average of 40 barrels, where the value is average % sugar ± standard deviation of 10 samples (No. 1 to 10).

Table 7. CARBOHYDRATE ANALYSIS OF DIFFERENT DRUMS FROM THE SAME PRODUCER.

Drum No.	% Fructose	% Glucose	% Sucrose
1	36.8	33.1	4.2
2	36.8	33.6	4.6
3	35.6	33.9	3.2
4	35.4	31.7	3.4
5	36.8	32.7	3.0
Average % sugar	**36.3**	**33.0**	**3.7**
Standard Deviation (σ)	**0.64**	**0.77**	**0.61**
Coefficient of variance (%)	**1.76**	**2.33**	**16.6**

Therefore, it was possible to utilize the knowledge of the sugar content of individual barrels to manipulate and adjust the content of the final product. Unfortunately, blending of the barrels still does not help to ensure a liquid product since all the crude samples have over 32 % glucose and, therefore, have a high tendency to crystallize.

In addition, all the final processed honey samples have approximately 33 % glucose and 38 % fructose (Table 6). The range of variation between the samples is very small. Yet, when these three samples (FH-1, FH-2, FH-3) were stored at 4 °C, they crystallized at different rates. Since these processed samples have similar carbohydrate composition and were processed from the same raw tank, this difference in crystallization rate must be due to factors other than composition. This observation revealed the necessity for a closer examination of the processing technique.

D. EFFECT OF FILLING TEMPERATURE.

In the processing plant, the incoming crude honeys are stored at 32-33 °C (90-92 °F). They are then mixed, and pasteurized at 85-88 °C (185-190 °F) at the rate of 0.756 kg/s (100 lb/min). The honey is filtered, cooled to 60-63 °C (140-145 °F) and kept in a large tank until filled into retail-sized bottles. Depending on how fast the samples are bottled, the temperature of honey during filling ranges from 60 °C to as low as 30 °C. This prompted a study of the effect of filling temperature on the rate of crystallization of the bottled samples.

Processed honey samples were collected and the filling temperature was noted. These samples were stored at 14 °C (the temperature at which crystallization is most rapid, Crane, 1975) for six months, followed by storage at 25 °C for another six months. Table 8 shows the analytical data on these processed honey samples and the degree of crystallization noted after the first six months and after a year. A result of 1 % crystals indicates presence of a few crystals (1-10) at the bottom of the container. Higher percentages of crystals were calculated as the height of the layer of crystals/height of honey in the container x 100. All carbohydrate determinations were performed on the acid resin column.

Viewing Table 8 and Figure 4, it is clear that the filling temperature is an important consideration in preventing later crystallization of the product. All samples filled while at temperatures between 30 and 40 °C showed appreciable crystallization after one year of storage, while samples filled at temperatures higher than 45 °C remained liquid. As mentioned earlier, the carbohydrate composition of all these samples was almost the same. Therefore, differences in crystallization appear to be due solely to differences in temperature of filling. However, these samples lost about 1% water after storage for a year and this would help hasten the crystallization process (discussion on moisture loss later) . No correlation could be found between the rate of crystallization and other factors like height of container or total weight of honey (Tables 8 and 9).

Table 8. COMPOSITION OF PROCESSED HONEY SAMPLES.

Filling Temp. (°C)	"Sucrose[1]" %	Glucose %	Fructose %	Fructose/ Glucose	Moisture[2] %	Crystals[3] %	
						6 months	1 year
33.3	7.8	34.9	37.9	1.09	17.1	5	11
33.3	7.5	33.9	38.3	1.13	17.4	6	12
35.0	7.9	34.2	38.4	1.12	17.4	5	35
35.6	7.1	34.9	38.4	1.10	17.9	15	17
35.6	7.9	33.8	39.1	1.16	17.7	3	32
37.2	7.4	34.8	38.6	1.11	17.4	2	21
37.8	8.0	34.3	38.4	1.12	17.4	11	17
42.2	8.3	33.7	37.5	1.11	16.9	1	10
42.2	7.4	33.6	36.7	1.09	17.0	0	0
47.8	7.5	34.8	38.1	1.09	17.4	0	0
47.8	8.1	34.7	38.1	1.10	16.9*	1	1
48.9	7.7	35.1	38.4	1.09	17.4	0	0
48.9	8.7	33.5	37.1	1.11	16.6	0	12
48.9	7.4	33.3	36.5	1.10	17.6	0	0
50.0	8.7	33.1	36.9	1.11	16.9	0	0
50.6	7.3	33.1	36.3	1.10	16.2*	0	0
51.1	9.4	33.5	37.8	1.13	16.6*	0	0
51.1	8.4	34.0	37.8	1.11	16.9	1	1
51.1	3.6	34.3	38.1	1.11	17.7	1	5
51.1	7.7	33.9	37.4	1.10	17.4	0	0
51.7	8.9	33.0	37.2	1.13	16.9*	1	2

52.2	7.9	34.9	38.1	1.09	17.0	0	0
52.2	7.8	33.7	37.5	1.11	17.4	0	1
52.2	9.0	33.9	37.6	1.11	16.6	0	0
53.3	9.2	33.4	37.9	1.13	17.0*	0	1
53.3	7.5	34.3	36.1	1.05	16.7	0	1
55.6	8.4	33.9	38.1	1.12	16.2*	2	2
56.7	7.1	34.6	37.8	1.09	16.7	1	1
58.9	7.3	33.3	36.6	1.10	17.7	0	0
60.0	7.2	34.9	37.8	1.08	16.7	0	0
AVERAGE:	**7.8**	**34.1**	**37.7**	**1.11**			

1. All analyses were performed on the acid cation-exchange resin column, therefore apparent sucrose includes all dissacharides.

2. Moisture levels after one year of storage: * = original moisture levels of $\leq$ 17.8 %; all others had original moisture levels $\leq$ 18.6 %.

3. Percentages reported as (height of crystals/height of honey in container) x 100. All samples were originally processed in January 1989.

Figure 4. EFFECT OF FILLING TEMPERATURE ON CRYSTALLIZATION.

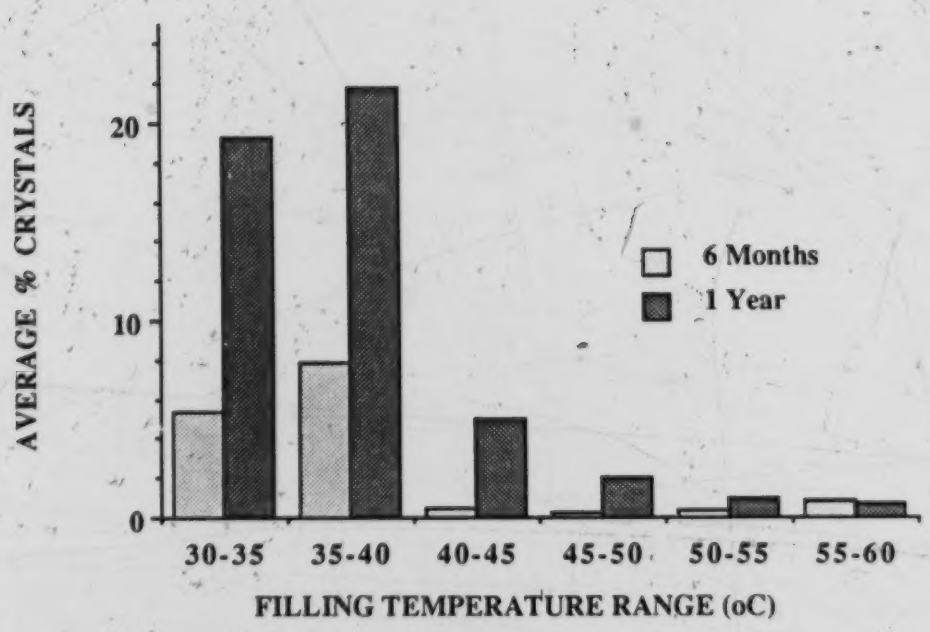


Table 9. PACKAGING SPECIFICATIONS OF THE PROCESSED HONEY SAMPLES

Temp.[1] (°C)	Total Honey Wt. (g)	Height Honey (cm)	Packaging Material[2]
33.3	552.4	11.5	PETG
33.3	482.8	11.0	LDPE
35.0	491.1	11.0	LDPE
35.6	539.2	11.0	LDPE
35.6	1068	17.0	PETG
37.2	488.3	11.0	LDPE
37.8	514.1	11.0	LDPE
42.2	1084	17.0	PETG
42.2	527	11.0	LDPE
47.8	493.1	11.0	PETG
47.8	810.3	13.5	PETG
48.9	485.5	11.0	LDPE
48.9	1065.7	17.0	PETG
48.9	539.9	11.5	LDPE
50.0	820.2	14.0	PETG
50.6	544.1	11.5	PETG
51.1	1047.6	17.5	PETG
51.1	823.9	13.5	PETG
51.1	533	11.5	LDPE
51.1	485.5	11.0	LDPE
51.7	1060.5	17.5	PETG

52.2	490.3	11.0	LDPE
52.2	478.3	11.0	LDPE
52.2	815.4	13.5	PETG
53.3	1049.2	17.0	PETG
53.3	809.9	13.5	PETG
55.6	277.3	9.5	PETG
56.7	1055.7	16.5	PETG
58.9	486.1	11.0	LDPE
60.0	801.5	14.0	PETG

1. See Table 7 for carbohydrate composition and degree of crystallization.

2. LDPE = low density polyethylene; PETG = Polyethylene teraphthalate.

The fact that filling the honey while hot keeps it liquid for a longer period could be due to several factors. It is known that merely heating the honey keeps it liquid for a longer period. On the other hand, the main factor could be that a sample that had started to cool in the large tank would be more easily induced to crystallize by the agitation that occurs during filling.

Heating the honey during pasteurization creates a metastable state. Solutions that are maintained at a temperature sufficiently higher than the saturation temperature for several hours have broader metastable zones (therefore, crystallize at a slower rate) than solutions whose temperature has not increased much above the saturation temperature (Nyvlt et al. 1985; Mullin, 1972). It should be stressed, however, that the initial processing of all samples was identical. The only difference was that some samples cooled more in the bulk tank before flowing into the individual containers.

The high temperature also affects viscosity of the honey. Munro (1943) measured the viscosity of different honeys including sweet clover and white clover. He used a MacMicheal viscometer to measure viscosities at different temperatures by the angular twist or torque of a standardized wire and converted these measurements to poise by multiplying the degrees recorded on the viscometer by the conversion factor of each wire. The wires were standardized against a 60 % w/w aqueous sucrose solution (for which viscosity is known). He observed that honey undergoes three successive stages of decreasing viscosity with rising temperature. As cold honey is warmed, it undergoes the most extensive decrease in viscosity per degree rise in temperature. Then there is a rapidly changing rate which terminates as honey becomes sufficiently reduced in viscosity for efficient handling (about 30 °C). Further rise in temperature results in only slight decrease in viscosity. Therefore, a change in temperature from 40 to 30 °C has a much greater effect on viscosity, and hence on crystallization, than a change from 60 to 50 °C.

In addition, the temperature of the honey during filling affects its ability to flow smoothly into the container. The increase in temperature, and subsequent decrease in

viscosity allows the honey to flow smoothly. This would prevent air bubbles from being trapped at the sides, between the honey and the wall of the container, and acting as nucleators. Also, if the honey is hotter and less viscous, air bubbles that form have a greater opportunity to escape to the surface. Several observations support this bubble theory. The appearance of crystals next to bubbles in honey jars indicates that the presence of bubbles can affect crystal formation. Also, honey producers have observed that injection of steam into containers and degassing heated honey prior to filling are procedures that seem to delay the crystallization of liquid honey. Both processing modifications would help prevent bubble formation during filling.

E. MOISTURE CONTENT.

Moisture content is very important in both liquid and processed crystallized (creamed) honey. A variation of 1% moisture in liquid honey can influence the rate of crystallization (Crane, 1975). The higher the moisture content the less the chance of crystallization. The amount of moisture present in the honey varies depending on the origin of the nectar and weather conditions. Generally, it ranges from 15-25 %, but Alberta honeys do not have moisture contents over 19%. Moisture has a profound effect on physical properties of honey such as crystallization, fermentation, viscosity and specific gravity (Gojmerac, 1980)

Several methods are available for determination of moisture content in honey. (Wedmore, 1955; Crane, 1975). Drying of the honey is performed at reduced temperature with reduced pressure (70 ^{o}C in vacuo) followed by measurement of weight loss. During this process, sand is added to increase bulk and porosity of the mass. Moisture content has also been determined by distillation with turpentine and measurement of the amount of recovered water. Other methods are the Karl Fischer chemical titration of water, density determination using a pycnometer, and the use of viscosity as a measure of moisture content (Wedmore, 1955). However, the most widely used method for determination of

moisture content in honey is the measurement of refractive index using a refractometer followed by conversion of the measurement obtained into % water using tables available in the literature (Crane, 1975; AOAC, 1990b). These tables were set up using honey of average composition. Although the composition of our honey samples may differ from those of average composition, all our samples have similar carbohydrate content and, therefore, there is minimum error in the use of the literature tables for comparison of the moisture content of the samples.

Determination of the moisture content of the crude honey samples after storage for one or two years at room temperature revealed that these samples lose about 1% water per year of storage (Table 5). Since honey is quite hygroscopic, this moisture loss was surprising. Some of the samples had less than 13% water after two years of storage, which is a very low moisture level. However, these samples were contained in bottles which were generally less than half-full. This may have increased the chances of moisture loss. Also, since these samples were up to two years old, their moisture level did not represent the original water content of the samples at the time of production. Processed liquid honeys stored at room temperature for one year also had about 1% less water than expected levels. The processed samples were all prepared initially to meet two moisture specifications; most of the samples (Table 8) had an original moisture content equal to or slightly less than 18.6 %, while the indicated samples were prepared to meet a lower moisture specification of 17.8 %. After about one year, all samples had lost significant amounts of moisture (Table 8). This moisture loss would aid crystallization.

The loss of 1 % water per year of storage is quite significant, since this means a loss of about 5 g of product for a 500 g bottle of honey, which could cause label non-compliance problems. This loss of moisture could be due partly to the low relative humidity in the Province. It is known that the optimum relative humidity for maintaining a 17.8 % moisture content in honey is about 60 % (Crane, 1975), and for maintaining a 17.4 % moisture is 58 % (Gojmerac, 1980). Honey exposed to lower relative humidity dries at a

faster rate. The lower the moisture content, the higher the chance of crystallization, as indicated by the fact that honeys with a moisture content of 20 % remain liquid for a very long time (Crane, 1975). However, the sample bottles were sealed throughout the period of storage, so that none of the moisture loss was through exposure to the environment.

Therefore, a closer look at the material from which the honey bottles are made is important. The bottles used for storage of the samples studied were made of either low density polyethylene (LDPE) or polyethylene terephthalate (PETG, Table 9). These materials are both generally regarded as good water barriers (Palling, 1980). Low density polyethylene is flexible, therefore used for squeezable bottles. It has a water permeability value of 1 g/mil-24 h-m^2 at 38 °C and 80 % relative humidity. PETG has good strength and transparency and has a water permeability of 4 g/mil-24 h-m^2 at 38 °C and 80 % relative humidity and a value of 6 g/mil-24 h-m^2 at 23 °C and 50 % relative humidity (Morris, 1986). In our case, the LDPE bottles were 1.0 mm thick, while the PETG containers were 0.8 mm thick. On average, the moisture loss from the 13 samples in LDPE containers was 1.2 %; while the average loss from the 17 samples in PETG containers was 1.5 % in one year. Using the above moisture permeabilities, the theoretical moisture loss possible is considerably higher for each material.

Further investigation into the apparent moisture loss and other possibilities for packaging materials (Mathlouthi, 1986) may be necessary to aid in the control of crystallization in liquid honey.

CONCLUSION.

The cation exchange resin in the H^+ form is a very convenient and useful column for analysis of carbohydrates at room temperature, while the resin column loaded with lead ions causes slight decomposition of the monosaccharides. All honey samples studied have more than 30 % glucose, therefore are supersaturated with glucose, but the glucose/fructose ratios in these samples are all very consistent which limits their use in

predicting crystallization rates, as recommended in the literature. However, crystallization of these samples could be delayed by filling the containers while the product is still hot (> 45 oC). Moisture loss was also noted in the plastic containers and this would contribute to crystallization (Assil et al. 1991).

CHAPTER 3

ANALYSIS OF FUMAGILLIN IN HONEY

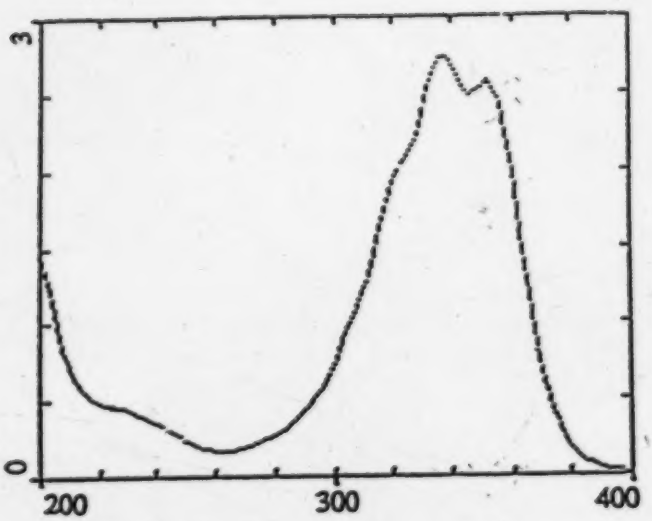
Several assay methods have been developed for fumagillin, including thin layer chromatography (TLC, Ochab, 1970; Isaaq et al., 1977), a microbial assay (Girolami, 1963), spectrophotometry (Garrett and Eble, 1954; Girolami, 1963), and a high performance liquid chromatographic method (HPLC, Brackett et al., 1988). However, most of these methods have disadvantages limiting their utility in the analysis of fumagillin and none has been applied to extensive analysis of fumagillin in honey produced from bees that were treated with the antibiotic.

TLC provides a rapid method of identification. Ochab (1970) developed solvent systems to distinguish fumagillin from other polyene antibiotics like amphotericin A and B and natamycin. The best solvent systems were one containing ethanol : ammonia : water : dioxane (8 : 1 : 1 : 1) and another made up of n-butanol : pyridine : water (15 : 10 : 5). Although TLC is useful for detection of the antibiotic, it provides only a limited amount of quantitative information.

The microbial assay detects the activity of fumagillin on a *Staphylococcus aureus* phage (Girolami, 1963). It is an effective method of analysis, but the procedure is long and tedious, requiring approximately 16-18 h for each analysis. In addition, microbiological assay procedures are generally nonspecific and the organisms used in the assay may respond to more than one antibiotic family, as well as other nonspecific inhibitory materials, especially in complex samples. In most cases, reasonably elaborate separations are needed to separate the various antibiotic families prior to analysis (Dixon et al., 1986).

The spectrophotometric assay is widely applied for analysis of fumagillin (Girolami, 1963). The ultraviolet (UV) spectrum of pure fumagillin exhibits maximum absorption at 335 and 351 nm (Figure 5). Although the higher wavelength, 351 nm, is

Figure 5. **UV SPECTRUM OF PURE FUMAGILLIN IN ACETONITRILE SOLUTION (24.0 mg/mL).**



used for the quantitative assay of the antibiotic, this method of analysis is still subject to error due to interferences from UV- absorbing impurities, especially in complex samples.

An HPLC method for analysis of fumagillin was reported by Brackett et al. (1988). It is easier and more accurate than the other available methods, and, therefore, we decided to adapt it to our honey system. In addition, a more sensitive and rapid screening method is required for detection of this antibiotic in honey samples. Hence, an enzyme-linked immunosorbent assay (ELISA) was developed for fumagillin in honey.

A. HPLC ANALYSIS OF FUMAGILLIN IN HONEY.

Brackett et al. (1988) developed an HPLC method for detection and quantitation of fumagillin using reversed-phase chromatography. The antibiotic was dissolved in acetonitrile and analyzed on an IBM C_{18} (5 mm, 15 cm x 4 mm i.d.) column at ambient temperature using acetonitrile : water : glacial acetic acid (500 : 500 : 1.5, v/v/v) as eluent (Brackett et al., 1988). The packing material in the C_{18} column is an octadecylsilyl phase in which silica is bonded to an 18-carbon alkyl chain. Separation of the components of the analyte is a result of hydrophobic interaction between sample molecules and the carbon chains on the silica surface. Since the octadecylsilyl surface is non-polar, the most polar sample components elute first and the least polar are the most retained on the column. Adsorption on the polar free silanol groups and partition between the mobile phase and the adsorbed layer of solvent also play a role in the separation.

For analysis of our honey systems, the honey was dissolved in acetonitrile : water (1 : 1, 5 g in 50 mL) and filtered through a membrane filter. The sample was injected through a 20 µL loop for analysis on a reversed-phase silica column (Phenomenex IB-SIL 5 C_{18} 150 x 4.6 mm) which was preceded by a guard column (Phenomenex IB-SIL 5 C_{18} 50 x 2.1 mm) and operated at ambient temperature. Figure 6 shows a typical HPLC chromatogram obtained for pure fumagillin dicyclohexylamine salt in CH_3CN : H_2O (1 : 1), using the C_{18} column and UV detection at 350 nm.

Figure 6. **HPLC CHROMATOGRAM FOR PURE FUMAGILLIN.**

Column **Phenomenex IB-SIL 5 C_{18};**

Eluent**: water : acetonitrile : acetic acid (500 : 500 : 1.5);**

Detection**: UV 350 nm.**

Scale denotes retention time in minutes.

HPLC was found to be a useful method in detecting fumagillin in honey samples. When a solution of fumagillin in acetonitrile : water (1 : 1) was added to the honey samples (dissolved in the same solvent) and analyzed, the detection limit was found to be 0.1 ppm of fumagillin in honey (gave peak height about 3 times noise level). The UV spectrum of fumagillin in honey (Figure 7) indicates that honey has a maximum absorption at 216 nm and there is little interference in UV detection between honey and fumagillin at 351 nm.

Peak areas correlated directly with fumagillin amounts to a concentration of at least 0.62 mg/mL of acetonitrile : water (1 : 1). Samples were analyzed in triplicate with an average coefficient of variation of $\pm$ 2.3 %.

Four honey samples, obtained from a research facility that regularly uses fumagillin as medication for its bees, were analyzed using the HPLC method. No fumagillin could be detected in any of the samples.

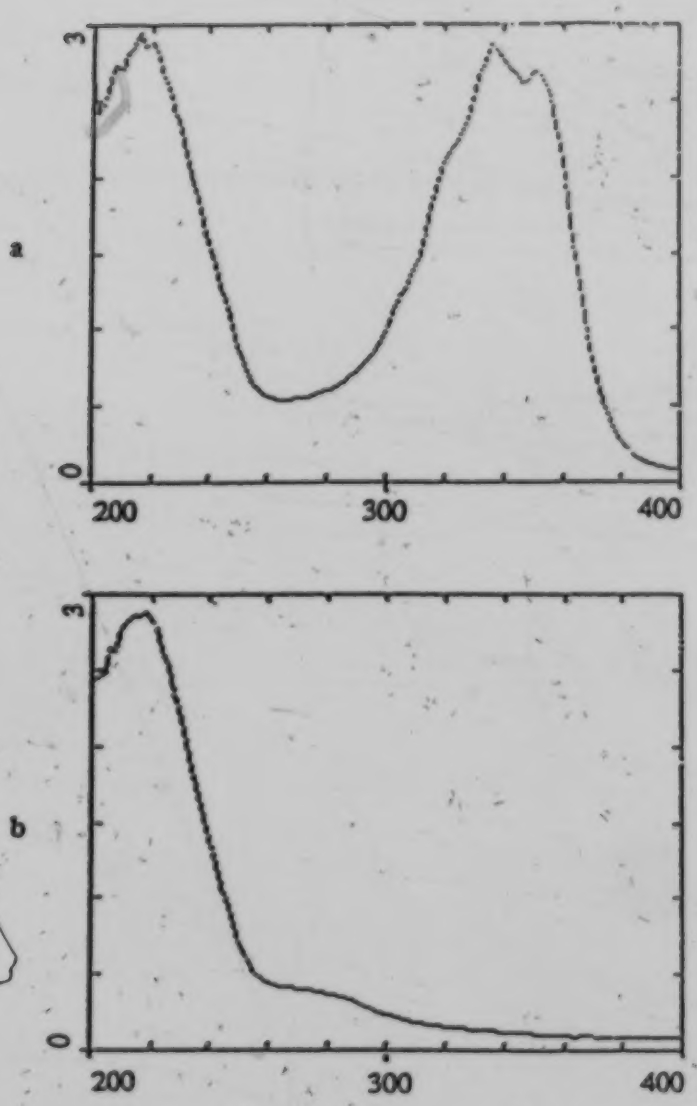
B. ENZYME-LINKED IMMUNOSORBENT ASSAY FOR FUMAGILLIN IN HONEY.

Although HPLC is an easy and reasonably sensitive technique, immunoassays are usually much more sensitive and specific and are very useful for rapid screening of a large number of samples in a relatively short time. Therefore, an ELISA was developed for detection of fumagillin in honey.

Preparation of protein-fumagillin conjugates.

Since fumagillin (FA) is a small molecule, it would not be immunogenic on its own and should be attached to a protein carrier. The stability and solubility of the hapten and nature of the groups available for conjugation determine the kind of reaction and the yields obtained in conjugation to a protein. In all proteins, the same functional groups are available for attachment to the hapten: the carboxyl groups of the C terminal and of the aspartic and glutamic acid residues, the amino groups of the N terminal and the lysine residues, the imidazo and phenolic functions of the histidine and tyrosine residues,

Figure 7. UV SPECTRUM OF (a) FUMAGILLIN IN HONEY 0.62 mg fumagillin/g honey (38.9 mg honey/mL; 24.0 mg fumagillin/mL) (b) PURE HONEY (38.9 mg/mL)



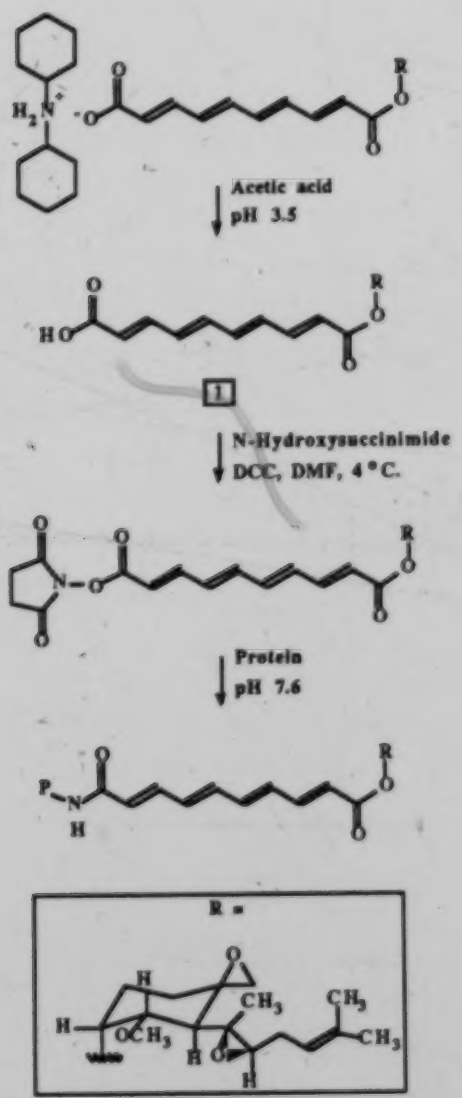
respectively, and the sulfhydryl group of cysteine residues (Erlanger, 1980). The major reactions used for protein modification are mainly acylating, alkylating, oxidizing, reducing, or electrophilic reactions (Kabakoff, 1980). For a hapten containing an acid group, like fumagillin, conjugation to a protein can be achieved via the formation of an active ester followed by an acylation reaction with the amino terminal of lysine residues in the protein (Anderson et al., 1964). Different methods for preparation of an activated acid derivative prior to coupling include mixed anhydride method, carbodiimide method, and N-hydroxysuccinimide ester formation (Kabakoff, 1980).

In the N-hydroxysuccinimide ester method, the acid and N-hydroxysuccinimide are reacted with a carbodiimide to form the active ester (Anderson et al., 1964; Bauminger and Wilchek, 1980). The activated carboxyl compound is then added directly to an alkaline-buffered protein solution. The acylation rate is pH dependent because the deprotonated lysine residues on the protein are stronger nucleophiles than their conjugate acid forms. Consequently, the reaction is usually carried out at pH 7.5 to 9.0. Since competition between protein derivatization and hydrolysis is a general problem with all acylating agents, excess acylating reagent is used in the reaction (Kabakoff, 1980).

To develop the ELISA for fumagillin, the acid was prepared from the commercially available dicyclohexylamine salt. Fumagillin (**1**, FA) was then attached to the protein via N-hydroxysuccinimide ester formation in the presence of dicyclohexylcarbodiimide (DCC, Scheme 3). Although this procedure gave satisfactory results with respect to the conjugate produced, solubility problems were encountered during the reaction of the active ester with the protein solution. This precipitation problem was also encountered by Goodrow et al. (1990). A water soluble carbodiimide like EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) is an alternative (Bauminger and Wilchek, 1980), although this would have led to protein cross-linking.

Generally, the protein carriers used in immunoassays include: globulin fractions, serum albumins (especially bovine serum albumin, BSA), hemocyanin (like *limulus*

Scheme 3. PREPARATION OF BSA-FA CONJUGATE.



polyphemus hemolymph, LPH), ovalbumin, thyroglobulin and fibrinogen (Erlanger, 1980). The protein bovine serum albumin (BSA) is quite stable and relatively resistant to denaturation under the chemical conditions needed to derivatize it (Kabakoff, 1980). *Limulus polyphemus* hemolymph (LPH, hemocyanin) is a completely foreign protein to rabbits and elicits high antibody formation (Nakashima et al., 1986). Therefore, BSA was used to prepare the conjugate with fumagillin (BSA-FA) used for coating the ELISA plates, while a fumagillin-LPH conjugate (LPH-FA) was injected into the rabbits to produce the antibodies.

Purification of the protein-hapten conjugate is generally straightforward since the reaction components differ markedly in molecular size. Gel filtration on Sephadex columns can be used, but the most common method is dialysis. Free hapten, excess reagents, and unwanted side products can be removed by placing the reaction mixture in a dialysis bag which allows only compounds of small molecular weights (< 12,000) to pass through the sides into the dialysis solution (Kabakoff, 1980). The protein-fumagillin conjugates were purified by dialysis in 8 M urea to allow the protein to unfold in order to remove any compounds that may be attached, not by covalent, but by hydrophobic or hydrogen-bonding. The urea solution was then replaced by an ammonium bicarbonate solution which evaporates during freeze-drying to leave the protein conjugate as a flaky material.

In ELISA techniques, bridge heterology (the use of different protein-hapten linking arms in the coating conjugate and the immunogen) is reported to significantly increase assay sensitivity (Tijssen, 1985). Antibody specificity is directed primarily at the part of the hapten molecule farthest removed from the functional group that is linked to the protein carrier (Erlanger, 1980). Nevertheless, antibodies are also raised against the linking arm. Therefore, they would have a higher affinity for a hapten bound to protein using a linking arm similar to that against which they were raised than to a different hapten-protein linkage. As a result, bridge heterology usually leads to a decrease in the difference between antibody

affinity for the bound hapten and the free hapten in the test sample, which allows increased competition by free antigen.

We, therefore, decided to prepare a coating conjugate with a different linking arm from that used in the immunogen in order to compare it to the conjugate BSA-FA, in which the linking arm is similar to that in the immunogen. Fumagillin (**1**, Scheme 4) was hydrolyzed to the alcohol fumagillol (**2**) and the polyene decatetraenedioic acid (**3**) under basic conditions (Landquist, 1956). The alcohol was then reacted with succinic anhydride in the presence of dimethylaminopyridine (DMAP) to produce the fumagillyl hemisuccinate (**4**) which was then conjugated to BSA via the N-hydroxysuccinimide ester to form the conjugate BSA-fumagillyl hemisuccinate (BSA-FS).

An important factor in the characterization and evaluation of protein-hapten conjugates is the determination of the protein to hapten ratio. For immunization, a certain number of hapten groups per protein molecule is optimal. About 15-20 moles hapten are usually attached per mole of protein in order to provide a wide variability of epitopes which results in sera with higher antibody affinity. Too much hapten on the protein surface lowers the variability in epitopes and, therefore, decreases the immunological response. However, the protein conjugate to be used as coating on the ELISA plate preferably contains a much lower hapten to protein ratio to encourage competition with the free haptens in the test sample and discourage multiple binding (avidity).

To determine hapten to protein ratio in a conjugate, spectrophotometry and elemental analysis are the most widely used methods. Fumagillin absorbs in the ultraviolet range and its spectrum (Figure 5) does not overlap with that of BSA (Figure 8). Therefore, UV was an ideal method to determine the number of fumagillin groups attached per BSA molecule. A standard curve, showing the absorbance of different concentrations of fumagillin in acetonitrile is shown in Figure 9. Absorption of the BSA-FA conjugate indicated that about two molecules of fumagillin were attached to each BSA molecule. UV

Scheme 4. PREPARATION OF BSA-FS CONJUGATE.

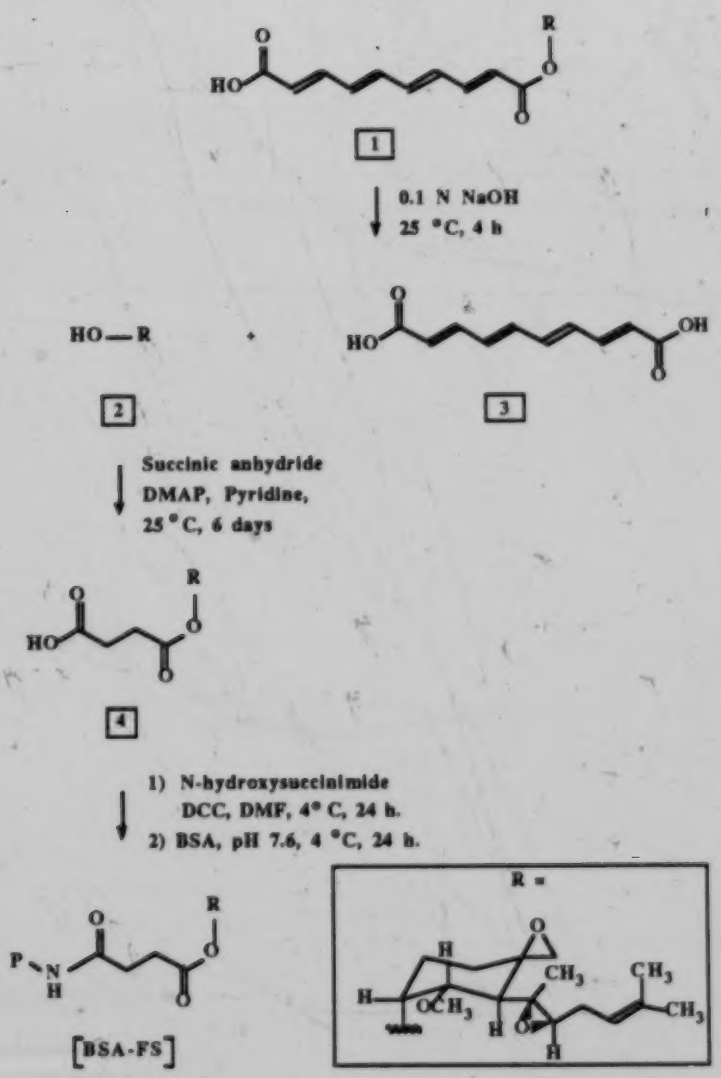


Figure 8 UV SPECTRUM OF BSA IN WATER (12.8 mg/mL).

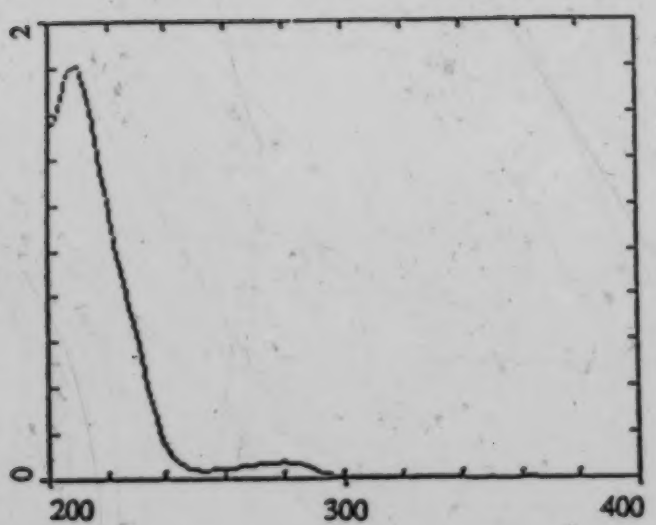
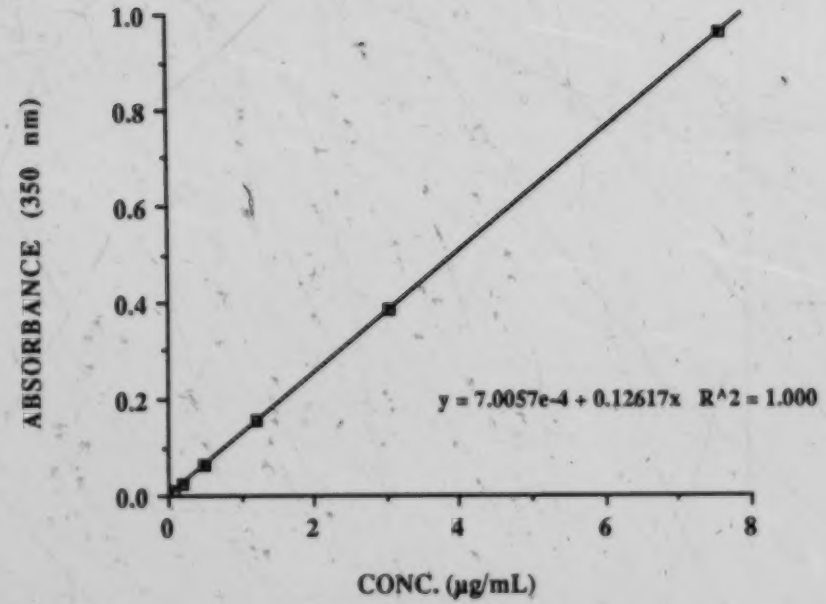


Figure 9. STANDARD CURVE FOR UV ABSORBANCE OF FUMAGILLIN SOLUTIONS IN ACETONITRILE AT 350 nm.



could not be used to analyze the LPH-FA conjugate because it was completely insoluble in all solvents. In this case, elemental analysis was very useful, especially since fumagillin contains no nitrogen. From elemental nitrogen analysis, the LPH-FA conjugate was found to have about 18 fumagillin molecules per protein.

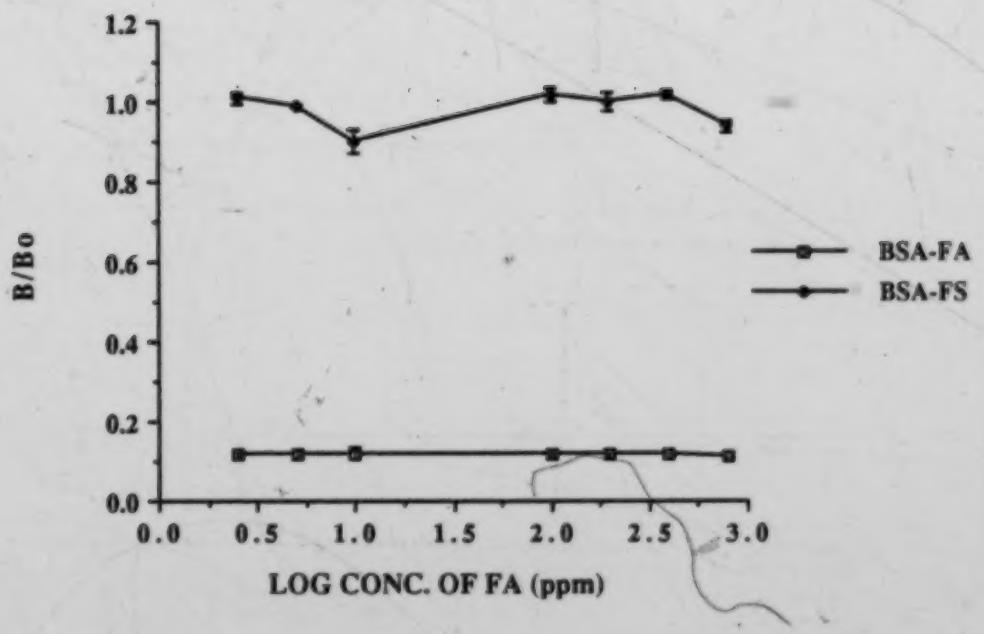
Comparison of BSA-FA vs. BSA-FS as coating conjugates.

The immunization conjugate LPH-FA was injected into two male rabbits and three boosting injections were given before the rabbits were bled 17 weeks later. The sera, containing the required antibodies, were collected and tested by a literature ELISA procedure (Sheth and Sporns, 1990) using either BSA-FA or BSA-FS as the coating conjugate on the microtitre wells.

Using BSA-FA conjugate (1 μg/mL in phosphate buffered saline, PBS) for coating, a 1000 times serum dilution was needed to get an O.D. of about 1 after half an hour, and a serum diluted 16,000 times gave 3 times the background absorbance. When BSA-FS conjugate (1 μg/mL in PBS) was used for coating, an O.D. of about 1 was obtained when the serum was diluted 2000 times, while a serum dilution of 16,000 times gave 30 times the background absorbance. Therefore, it appears that the antibodies bind more strongly to the succinyl conjugate than to BSA-FA.

When the sera were tested in an indirect competitive ELISA with different concentrations of free fumagillin, no significant reduction in absorbance could be detected with the BSA-FS as coating conjugate, even with test samples containing up to 800 ppm of free fumagillin in solution (Figure 10). This was a surprising result, but it may be accounted for by the difference in structure between fumagillin (**1**) and its derivative. The hemisuccinate (**4**) lacks the long rigid polyene chain, which is replaced by a shorter but more flexible methylene chain. This flexibility may allow the structure of the hemisuccinate to orient itself more favourably in the antibody binding site and cause the binding to be practically irreversible. Another reason could be the fact that BSA-FS contains about 14

Figure 10. **COMPETITIVE ELISA FOR FUMAGILLIN IN SOLUTION (ETHANOL : WATER, 1 : 4) USING BSA-FA (1.0 µg/mL) AND BSA-FS (1.0 µg/mL) AS COATING CONJUGATES - Serum diluted 1000 times. Error bars denote standard deviation of triplicate analyses. B = Absorbance for test sample; B_o = Absorbance for solution with no fumagillin.**



hapten molecules/protein, while BSA-FA contains only 2, and therefore, the increased binding with the BSA-FS conjugate is a reflection of multiple binding (avidity) by each antibody to the large number of available haptens coated on the plate.

The BSA-FA conjugate proved to be very useful in the development of a sensitive and effective indirect competitive ELISA for fumagillin. Figure 11 shows the results of a competitive ELISA using BSA-FA (1 μg/mL in PBS) for coating of the microtitre wells, a 1000 fold dilution of antibody sera and free fumagillin as a solution in ethanol : water (1 : 4) at different concentrations. Under these conditions, levels of fumagillin down to about 35 ppb of fumagillin in solution could be detected (20 % decrease in absorbance), and about 100 ppb resulted in a 50 % reduction in absorbance.

The BSA-FA conjugate used in all tests contained about 2 fumagillin groups per BSA molecule. Similar conjugates with more groups per protein resulted in less efficient competition. In addition, they had lower solubilities and could not be dissolved completely in any solvent.

ELISA for fumagillin in honey.

To analyze for fumagillin in honey, it is essential to limit the variability between different honeys that may be due simply to the variability in the nature of the honeys and not to different levels of fumagillin that may be present. Honey samples were diluted with water to different concentrations and analyzed using different serum concentrations in an attempt to optimize the conditions under which they can be analyzed by ELISA.

To determine the ideal concentration of honey to be used in the tests, a honey sample free of fumagillin was diluted to different concentrations with water and an ELISA was performed. The results (Figure 12) indicate that more concentrated solutions of honey are less likely to exhibit wide variabilities between different samples. Table 10 shows the results for ELISA on honeys spiked with known amounts of fumagillin. It was apparent that the use of a serum diluted 1000 times and a honey concentration of about 0.5 g/mL

Figure 11. **COMPETITIVE ELISA FOR FUMAGILLIN IN SOLUTION (ETHANOL : WATER 1 : 4) USING: BSA-FA for coating (1.0 µg/mL in PBS); serum diluted 1000 times.**

Error bars denote standard deviation of triplicate analyses.

B = Absorbance for test sample;

B_o = Absorbance for solution with no fumagillin.

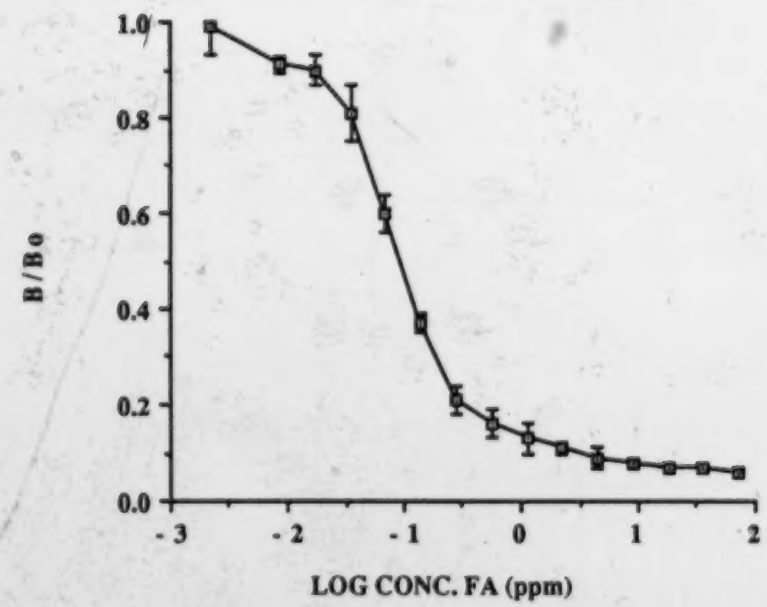
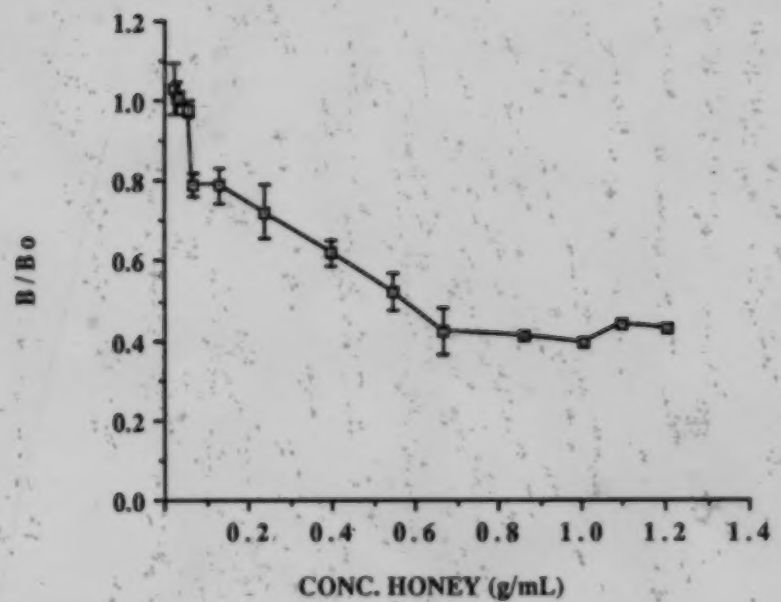


Figure 12. **B/Bo FOR A HONEY AT DIFFERENT CONCENTRATIONS IN ELISA: coating conjugate BSA-FA (1.0 μg/mL); serum dilution: 1/1000. Error bars denote standard deviation of triplicate analyses. B = Absorbance for test sample; B_o = Absorbance for solution with no fumagillin.**



leads to the most sensitive results. However, the absorbances obtained for such solutions are very low (an O.D. of about 0.1-0.4). Using a more concentrated serum (500 fold dilution) and the honey solution at a concentration of 0.5 g/mL seems to be the best condition. In this case, honey containing 10 ppb fumagillin would give B/Bo of about 0.5. It was interesting to note that the ELISA test was about an order of magnitude more sensitive when honey was present in solution than when only solvent was used (Figure 11).

Table 10. DETECTION LIMIT FOR FUMAGILLIN IN HONEY USING ELISA.

Case No.	Serum dilution	Honey dilution	Conc. FA in Honey (ppb) to give B/Bo ~ 0.5
1.	1/500	1/1	20
2.	1/500	1/2	10
3.	1/1000	1/2	5

To determine the variability between different honeys, a wide range of honey samples were tested by ELISA under different conditions (Table 11). The control honey, which was used as a reference to obtain Bo, was a sample obtained from a local producer known to avoid the use of any chemicals, including fumagillin. Honey samples M, N, O and P were provided by a honey producer that uses fumagillin as medication for his bees. These samples were the ones analyzed by HPLC and in which no fumagillin could be detected by that method. It is clear that there is least variability in the different honeys when the serum is diluted 500 times and the honey is at a concentration of 1 g/mL (case 1). Under these conditions, it can be stated that any honey that causes a 50% reduction in absorbance compared to a control honey may be suspected of contamination with

Table 11. ELISA OF HONEY SAMPLES PRODUCED IN ALBERTA.

Honey No.[1]	B/B_o[3]		
Case No.[2]	1	2	3
A*	—[4]	0.38	0.48
B	0.98	0.89	1.06
C	0.88	0.64	0.72
D	0.77	0.55	0.67
E*	0.73	—	0.48
F*	—	—	0.48
G	—	—	0.62
H*	—	—	0.50
I	0.77	0.58	0.73
J	0.96	0.82	0.61
K	1.06	1.02	—
L*	0.87	0.22	—
M	1.08	1.04	0.93
N	0.94	0.94	0.77
O	0.78	0.94	0.72
P*	0.73	0.88	0.25
Range of B/Bo	**0.7 - 1.1**	**0.4 - 1.0**	**0.3 - 1.1**

1. Honey M, N, O and P were produced by bees given fumagillin for medication; Honeys A to H were unprocessed honey samples isolated from individual combs obtained from individual producers in 1989; Honey I was a processed honey and a composite of honeys from different producers. Honeys J, K and L were unprocessed composites from individual producers. HPLC analysis of honeys L, M, N, O and P indicated no detectable (<100 ppb) fumagillin.

2. As in Table 10: Case No. 1: the serum was diluted 500 times, concentration of honey was about 1 g/mL; Case No. 2: serum dilution = 500 times, concentration of honey = 0.5 g/mL; Case No. 3: serum dilution = 1000 times, concentration of honey = 0.5 g/mL; Case No. 4: serum dilution = 1000 times, concentration of honey = 0.02 g/mL.

3. B = Absorbance for test sample; B_o = absorbance for control honey.

4. No values are available.

* Samples that <u>may</u> contain 5-10 ppb fumagillin.

fumagillin and, in that case, another method, like HPLC, may be used to further investigate that suspicion. However, the problem in dealing with a honey at such a concentrated level is related to its high viscosity which does not facilitate the handling and pipetting of the sample into the microtitre wells. Dilution of the honey by half renders it easier to handle. A serum diluted 500 times gives average absorbances of about 0.6 when the honey concentration is about 0.5 g/mL. Under these conditions, normal honeys give B/Bo ranging from 0.4 - 1.0. A honey sample that causes over 90 % reduction in absorbance with reference to the control honey is likely to be contaminated by the antibiotic.

All ELISA tests were performed in triplicate on the same plate. The maximum % relative standard deviation of triplicate analyses was 12 % (average value was 6 %).

A careful examination of Table 10 and 11 indicate that a few of the honey samples presented in Table 11 (indicated by *) could have contained 5 to 10 ppb of fumagillin (although no proof is available and the low absorbances for the honeys may be due to other factors). However, not only would this level of contamination be very difficult to confirm by HPLC (100 ppb detection limit), but also regulatory agencies usually consider levels of agricultural chemicals less than 100 ppb in a food tolerable.

From ELISA and HPLC analysis on the honey samples, it appears that fumagillin used as medication for the bees does not lead to contamination of the honey produced to reasonably detectable levels (20 ppb). This is a reasonable possibility, since the antibiotic is usually fed to the bees before the flow of nectar and may, in some way, be metabolized by the bees before the period of honey production. In any case, it does appear that the use of fumagillin as an antibiotic does not affect the safety of honey for human consumption.

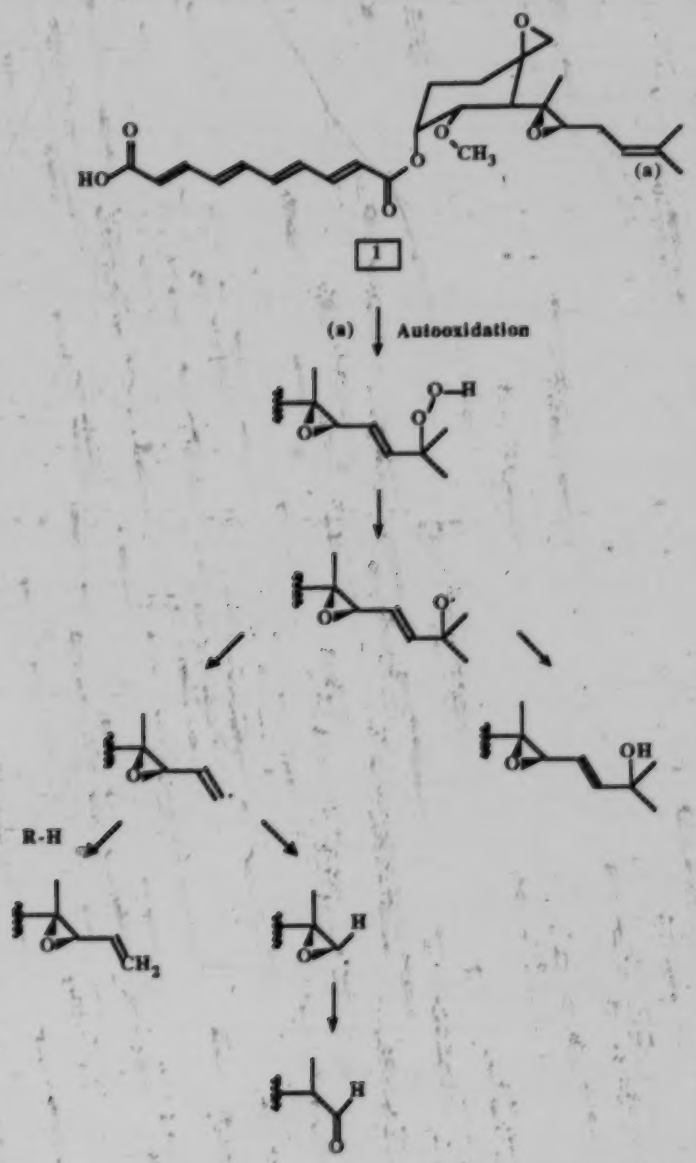
CHAPTER 4

STUDIES ON THERMAL AND PHOTOLYTIC DEGRADATION OF FUMAGILLIN IN HONEY USING HPLC AND ELISA.

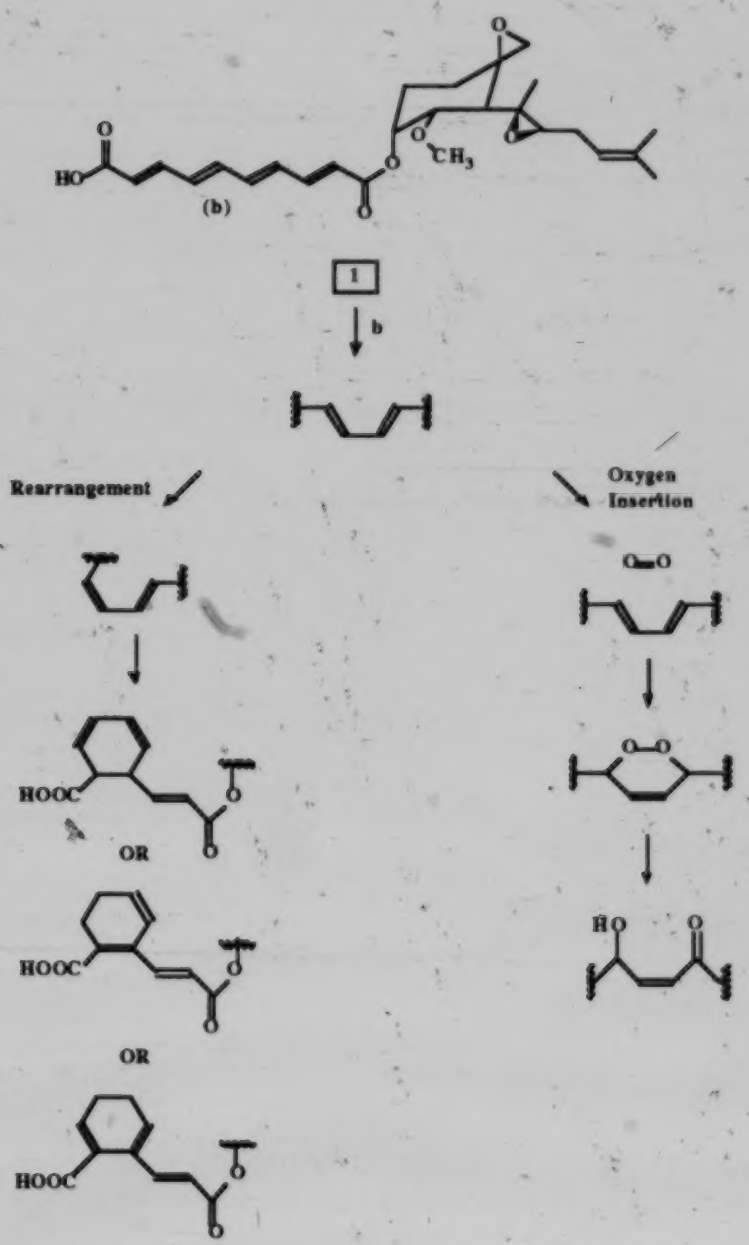
Fumagillin (**1**) has several potential labile sites that are susceptible to attack by nucleophiles in the presence of heat or light (Scheme 5a, 5b and 5c). In the presence of light, oxygen in the air may cause autooxidation of the unsaturated -C=C- bond with an adjacent allylic proton, leading to the formation of a hydroperoxide (Scheme 5a: March, 1985; Wong, 1989). Dissociation of the unstable -O-O- linkage may then lead to several possible products. Light may also cause the rearrangement of the conjugated tetraene chain (Scheme 5b). Insertion of an oxygen molecule into the unsaturated double bonds could then, in principle, lead to the formation of new carbonyl or hydroxyl functionalities on the polyene chain. Light can also catalyze the rearrangement of one of the double bonds from a trans to a cis conformation. This rearrangement could lead to the formation of cyclic derivatives, and this was the route of decomposition which Garret and Eble (1954) regarded as the most probable.

In acidic media, hydrolysis of the ester linkage is likely, especially at high temperatures (Scheme 5c), leading to the formation of fumagillol (**2**) and decatetraenedioic acid (**3**). Acid or heat may also facilitate the hydrolysis of the epoxides in the molecule. The less conjugated epoxide (d) is more reactive than the epoxide in the side chain (e) because it is more strained, due to its attachment to another ring, and is less sterically hindered to attack by nucleophiles. Tarbell et al. (1961) found that the epoxide in the side chain (e) is quite stable to several chemical reagents while the other epoxide (d) is very labile.

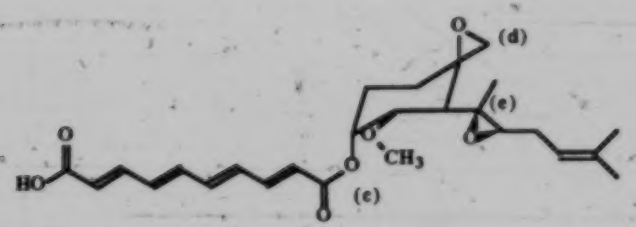
Scheme 5a. POSSIBLE ROUTES FOR DECOMPOSITION OF FUMAGILLIN IN LIGHT BY AUTOOXIDATION.



Scheme 5b. POSSIBLE ROUTES FOR DECOMPOSITION OF FUMAGILLIN IN LIGHT THROUGH REARRANGEMENT OF POLYENE CHAIN.



Scheme 5c. POSSIBLE ROUTES FOR DECOMPOSITION OF FUMAGILLIN IN HEAT.

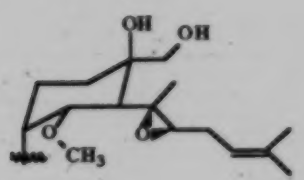


[1]

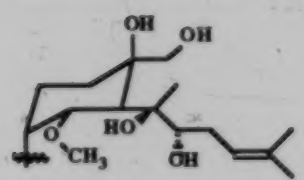
c ↙ Ester Hydrolysis

d, e ↘ Epoxide Hydrolysis

Fumagillol (2) + Acid (3)



OR



Honey is essentially a concentrated aqueous solution of sugars with a pH of about 3.5. During its production by the bees, it is stored in the hive, which is a dark enclosure maintained at a temperature of 37 °C. Processing of honey exposes it to light and pasteurization temperatures as high as 77 °C (but only for a few seconds). Therefore, to determine the route of decomposition of fumagillin in honey, in case the honey gets contaminated with the antibiotic during its production and storage, we decided to study the decomposition of fumagillin in the dark at temperatures ranging from 37-80 °C, and in light at 37 °C.

A. THERMAL DECOMPOSITION OF FUMAGILLIN

To determine the actual product of decomposition of fumagillin on heating, the compound was dissolved in 10% ethanol in water (acidified with dilute acetic acid to pH 3.5), protected from light, and heated at 60 °C. After 42 days, some fumagillin could still be detected by TLC. HPLC on the reaction mixture (Figure 13) indicated the presence of one major product which was isolated by flash chromatography. The spectra of the product **5** were compared to those of fumagillin and fumagillol. It appears that, on heating, the unstable epoxide in the molecule is hydrolyzed to give the dihydroxy product **5** (Scheme 6), but the exact stereochemistry of the product is unknown. No ester hydrolysis occured. This is evident from the fact that no fumagillol or decatetraenedioic acid were detected by TLC or HPLC.

Tarbell and co-workers (Cross and Tarbell, 1958; Tarbell et al. 1961) had reported the isolation of a crystalline compound resulting from the action of 10% sulfuric acid on fumagillol (**2**) with boiling under reflux. They observed that their product does not undergo hydrogenation and, therefore, must have lost the double bond in the side chain. They proposed two possible structures for their product resulting from the rearrangement of the dihydroxyfumagillol intermediate (Scheme 7). Presence of a strong IR band at 831 cm^{-1} was provided as possible evidence for presence of a $(CH_3)_2$-CO-linkage. No NMR spectra

Figure 13. **HPLC CHROMATOGRAM OF THE THERMAL DECOMPOSITION OF FUMAGILLIN IN SOLUTION.**

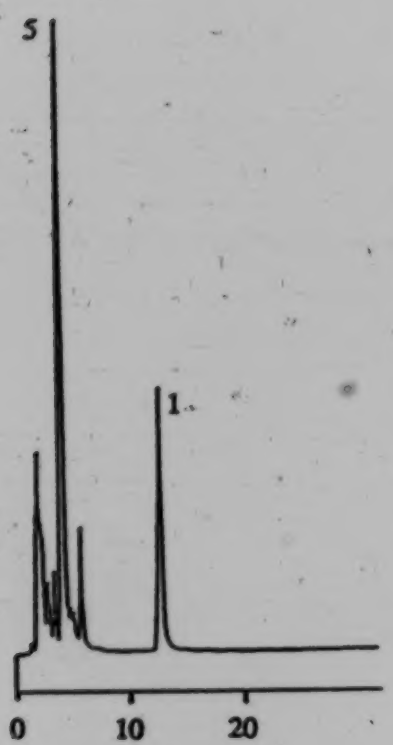
***Column*: Phenomenex IB-SIL 5 C_{18};**

***Eluent*: water : acetonitrile : acetic acid (500 : 500 : 1.5);**

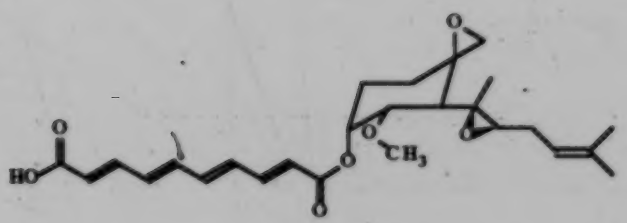
***Flow rate*: 1.0 mL/min. *Detection*: UV 350 nm.**

Scale denotes retention time in minutes.

1= fumagillin; 5= dihydroxyfumagillin (compound 5)

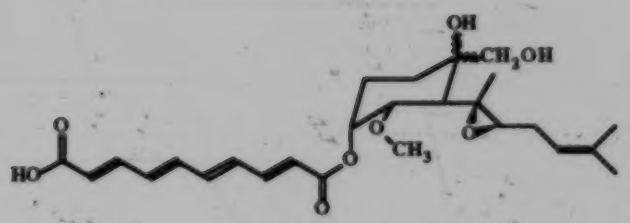


Scheme 6. THERMAL DECOMPOSITION PRODUCT OF FUMAGILLIN IN SOLUTION.



1

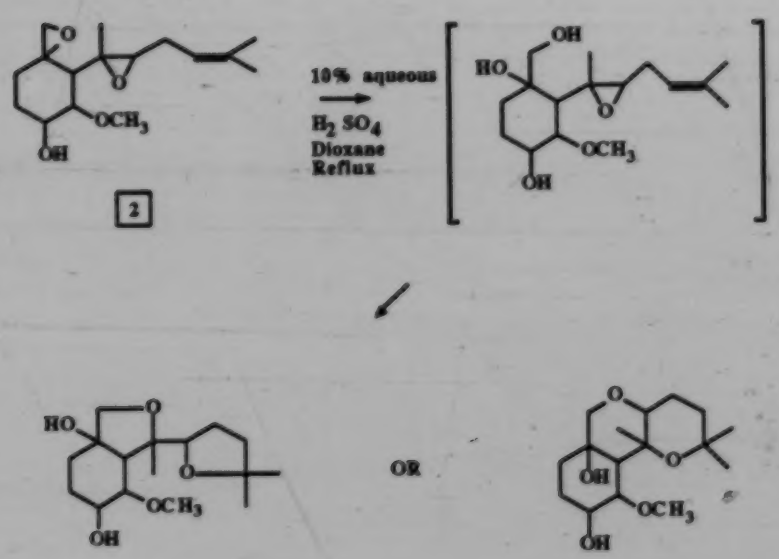
10 % Ethanol in water
pH 3.5, 60 °C, 42 days



5

were reported. However, in our case, the conditions used for the thermal decomposition of fumagillin are much milder than the conditions of Tarbell and co-workers (1961). In addition, the spectral data of **5** are more consistent with the structure indicated than with ring structures similar to those in Scheme 7. The IR spectrum of **5** is very similar to that of fumagillin except for the presence of the -OH band at 3431 cm^{-1}. No band at 831 cm^{-1} was observed, while the presence of bands at 1277, 880 and 850 cm^{-1} is probably due to the unreacted epoxide present.

Scheme 7. RESULTS REPORTED BY TARBELL ET AL. (1961) ON ACID HYDROLYSIS OF FUMAGILLOL (2).



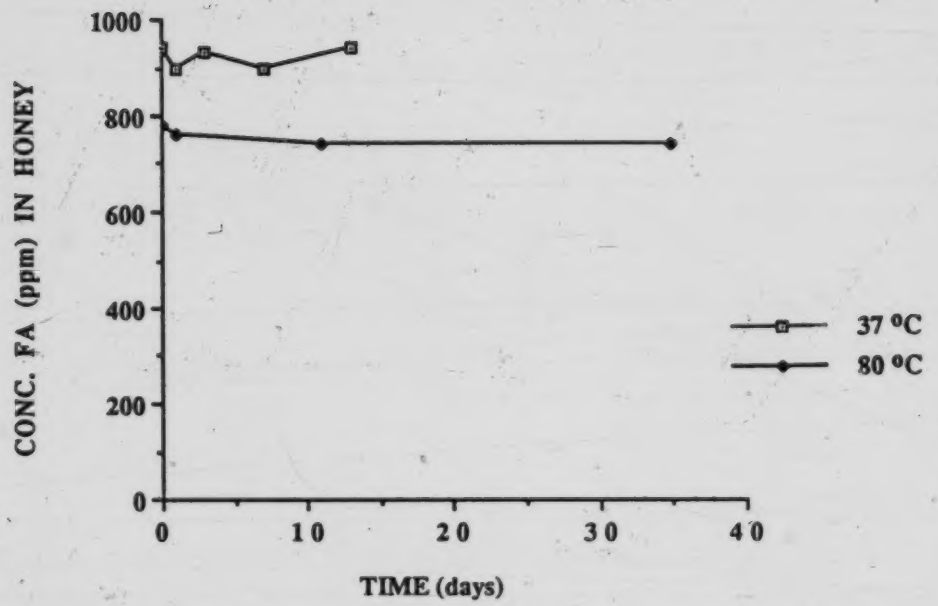
To determine the rate of decomposition of fumagillin in honey, two experiments were set up at different temperatures. Flasks containing honey spiked with fumagillin and

control samples of honey were protected from light and heated at 37 °C, or 80 °C. Samples of these solutions were removed at different intervals and tested by HPLC and by ELISA. As indicated in Figure 14, the amount of fumagillin present in the solution, as determined by HPLC, shows very little change over a period of up to 35 days at a temperature of 80 °C. This same trend was observed when ELISA was used to estimate the amount of fumagillin in the honey over the same period. This indicates that fumagillin is thermally stable in honey even at temperatures much higher than it is likely to be exposed to during production and processing. When ELISA was performed on the decomposition product **5**, a solution of 1.6 μg/mL of **5** gave about 25% reduction in absorbance with respect to the solvent blank. The same percent reduction in absorbance is observed for a solution of fumagillin containing about 50 ppb (Figure 11).

B. PHOTOLYTIC DECOMPOSITION OF FUMAGILLIN

A solution of fumagillin in acetonitrile : water (1 : 1) was exposed to sunlight and fluorescent light. After 7 days, one major product could be detected by TLC. However, attempts to isolate and purify the compound led to some decomposition, and the compound was purified by preparative TLC. The spectral data of the product **6** indicate that some rearrangement of the polyene chain may have occurred (Scheme 8). This is in accordance with the observation made by Garrett and Eble (1954) who named the product "neofumagillin" and observed that it absorbs at wavelengths less than 345 nm and that, on further exposure to light, it undergoes rearrangement to the structures shown in Scheme 5b. Garret and Eble's observations were based on kinetic and UV studies of the photolytic decomposition of fumagillin in ethanol and not on isolation or spectral analysis of products. Compound 6 has an NMR almost identical to that of fumagillin except in the region of -C=C-H protons where only 6 vinyl protons appear in the region of δ 7.2-5.8, instead of the 8 protons of the polyene chain in fumagillin, and two extra protons appear in the region for R-C-H protons (δ 2.1-1.8). IR spectrum of **6** lacks the peak at 1627 cm^{-1} which is

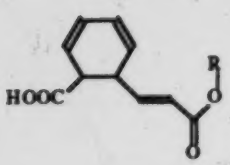
Figure 14. THERMAL DECOMPOSITION OF FUMAGILLIN IN HONEY WITH TIME AT 37 °C AND 80 °C - MEASURED BY HPLC.



Scheme 8. DECOMPOSITION PRODUCT OF FUMAGILLIN IN LIGHT.

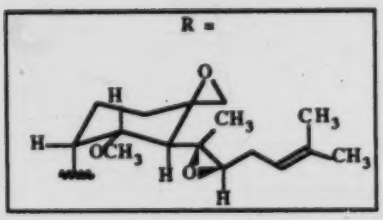
1

Light
Solvent



"Neofumagillin"

6



present in fumagillin (for conjugated -(C=C)$_4$- bonds) and exhibits a new peak at 1617 cm^{-1}, which may be assigned to the new -C=C- bonds.

In the ELISA, the antibodies recognize compound **6**. A solution containing 2.0 µg/mL of **6** causes a 92% reduction in absorbance with respect to a solvent blank. A fumagillin solution containing about 1 ppm of fumagillin in solution (Figure 11) caused about the same percent reduction in absorbance (the antibodies were about twice as sensitive for fumagillin as compound **6**).

To determine the rate of the photolytic decomposition of fumagillin in solution, fumagillin was dissolved in acetonitrile : water (1 : 1, 24 µg/mL). The solution was left in daylight/fluorescent light and tested periodically by HPLC. The results (Figure 15) indicate that, in light, fumagillin decomposes quickly in the first few hours. After about 5 hours, it begins to decompose more slowly. In about 11 days, the amount of fumagillin in the solution had decreased to about 30 % of its original level. The UV absorption spectra of the decomposing solution at different intervals is shown in Figure 16. It is obvious that, although much degradation occurs, it is not necessarily reflected in the UV absorption of the total mixture. Figure 17 shows the results obtained by HPLC on a solution of fumagillin in honey (0.62 mg FA/g honey in acetonitrile : water 1 : 1) in light. The decomposition of fumagillin in honey in light seems to be more gradual than in the absence of honey.

Another experiment was also performed where a flask containing honey and fumagillin and another flask containing control honey were left at 37 °C under intense light. Samples of the experiment and the control were checked periodically by both HPLC and ELISA (Figure 18). The HPLC chromatogram for the honey containing fumagillin after one day in intense light is given in Figure 19. After one day, HPLC indicated that only about 1/3 of the fumagillin remained (Figure 18). The major new peak (Figure 19) was neofumagillin (**6**). Since the UV maximum for 6 was at 240 nm rather than 350 nm (the optimum wavelength for fumagillin and at which the HPLC chromatogram was obtained)

Figure 15. PHOTOLYTIC DECOMPOSITION OF FUMAGILLIN IN ACETONITRILE : WATER (1 : 1, 24 μg/mL) - MEASURED BY HPLC

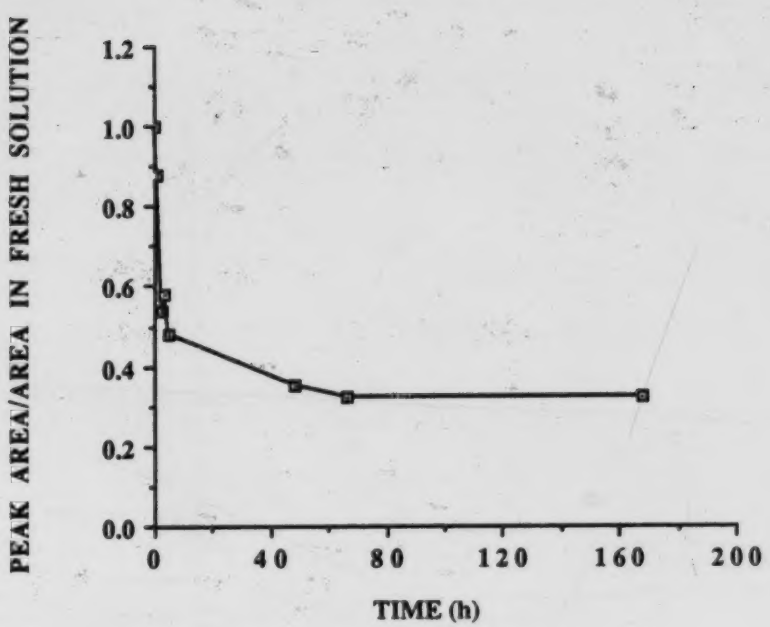


Figure 16. UV ABSORPTION SPECTRA OF FUMAGILLIN IN ACETONITRILE : WATER (1 : 1) DECOMPOSING IN LIGHT.

a Fresh solution

b 2 h in light

c 18 h in light

d 1.5 months in light

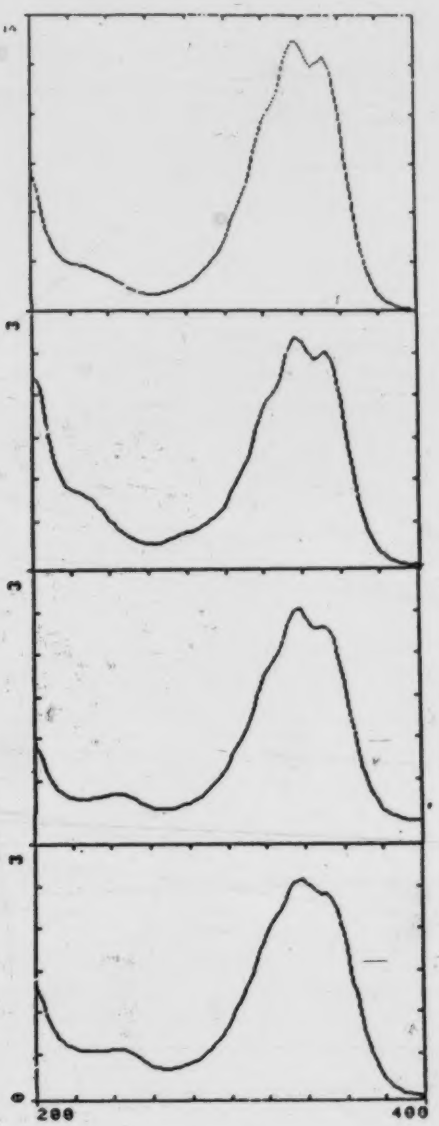


Figure 17. RATE OF PHOTOLYTIC DECOMPOSITION OF FUMAGILLIN IN HONEY - MEASURED BY HPLC.

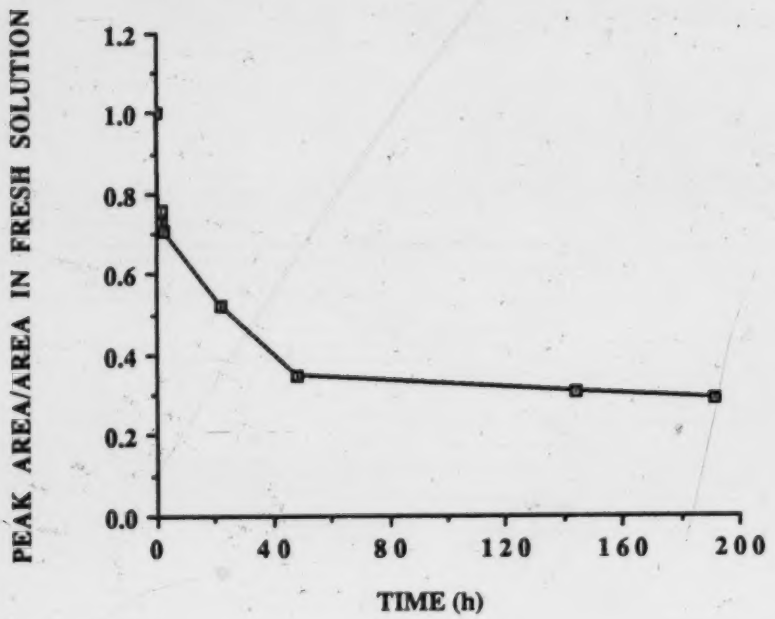


Figure 18. DECOMPOSITION OF FUMAGILLIN IN HONEY UNDER INTENSE LIGHT - MEASURED BY HPLC AND ELISA.

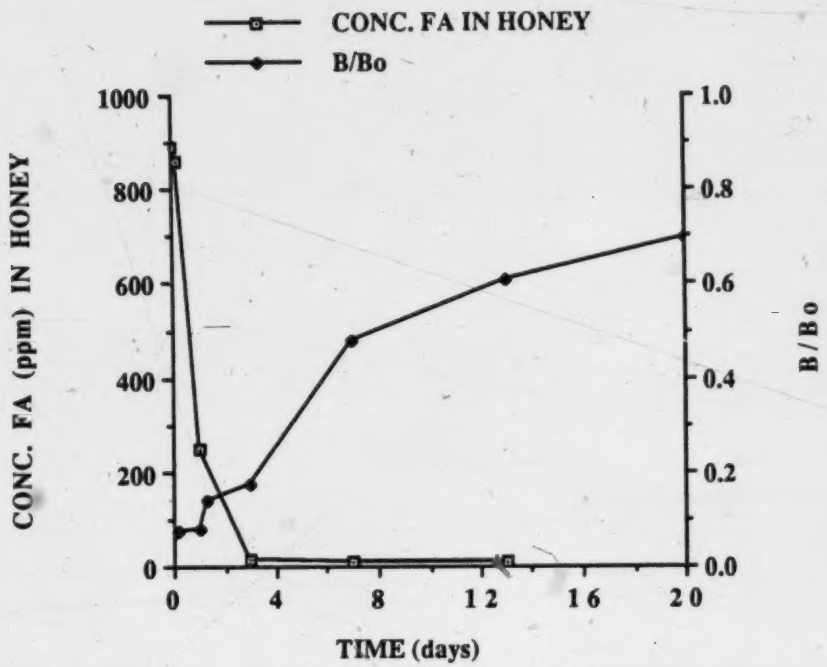


Figure 19. **HPLC CHROMATOGRAM FOR DECOMPOSITION OF FUMAGILLIN IN HONEY IN INTENSE LIGHT FOR 1 DAY.**

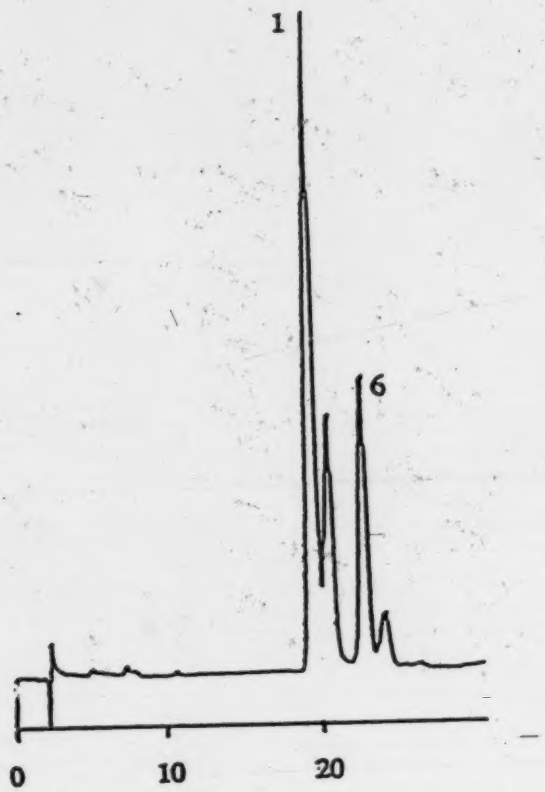
***Column*: Phenomenex IB-SIL 5 C_{18};**

***Eluent*: water : acetonitrile : acetic acid (500 : 500 : 1.5);**

***Flow rate*: 0.7 mL/min. *Detection*: UV 350 nm.**

Scale denotes retention time in minutes.

1 = Fumagillin; 6 = photolytic decomposition product.



the relative areas of the peaks for 1 and 6 do not reflect the actual ratio of the compounds present.

The ELISA results (Figure 18) indicated a less rapid increase in B/Bo than the decrease in fumagillin concentration noted by HPLC. This is due to the orders of magnitude greater sensitivity for fumagillin in ELISA, and also to the fact that the ELISA is sensitive to the presence of at least the major decomposition product **6**.

CONCLUSION

Two sensitive and effective methods for detection of fumagillin in honey have been developed. ELISA is a highly sensitive test that could become very useful for screening for fumagillin in a large number of honey samples in a relatively short time. It can also detect the photolytic decomposition product of fumagillin, if present. Since fumagillin is essentially thermally stable in honey at the temperatures it is likely to be subjected to during processing, the only decomposition product likely to be present is the product obtained as a result of exposure of honey to light during processing. ELISA is, therefore, the most reliable method developed so far for screening honey samples to ensure lack of contamination by the antibiotic.

HPLC is also very sensitive and requires minimum sample purification before analysis. It can also detect the decomposition products and can, therefore, be used as a method of confirmation of results for samples suspected to be contaminated.

Although no fumagillin has been detected in any of the honey samples tested (down to 20 ppb level), only four of the sixteen honey samples analyzed for fumagillin were from a producer that is known to use fumagillin as medication for bees. Therefore, a wider survey may be necessary to ensure that fumagillin fed to bees does not find its way into the honey produced. In any case, methods are now available to ensure the safety of honey to the consumer (Assil and Sporns, 1991).

CHAPTER 5

EXPERIMENTAL

GENERAL

Honey samples were provided by Alberta Honey Producers' Co-op. Ltd, Edmonton, Alberta. Samples of fumagillin dicyclohexylamine salt were obtained from Medivet Pharmaceuticals Ltd. N-hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide and N, N-dimethylformamide were purchased from Aldrich. *o*-Phenylenediamine tablets, goat antirabbit peroxidase conjugated antibodies, urea peroxide and citric acid monohydrate were obtained from Calbiochem Co. Thimerosal, Tween 20, BSA and LPH were purchased from Sigma Chemical Co. Microtitre plates for ELISA (Dynatech Immulon 1 microtitre plates) and spectrapor dialysis tubing (12,000-14,000 mw cutoff) were acquired from Fisher Scientific. Freund's complete and incomplete adjuvants were purchased from Difco Laboratories. Water used in all reactions and ELISA tests was purified using a Milli-Q system from Millipore. All other reagents used were reagent grade or better.

Absorbance values at different wavelengths were recorded with a Hewlett-Packard Model 8451 A diode array spectrophotometer. Optical densities in microtitre plate wells were measured with a Model EL 309 ELISA reader from Bio-Tek Instruments, Inc. Centrifugation was performed in a Damon/IES division model HN-S II centrifuge from International Equipment Co. Nuclear magnetic resonance (NMR) spectra were measured on Bruker WM-360 or WH-400 instruments. Tetramethylsilane was used as the internal standard. Peaks in the NMR spectrum of fumagillin were assigned to individual protons based on information obtained from ^{1}H-^{1}H decoupling experiments, some assignments reported by Tarbell et al. (1961), and basic NMR principles (Silverstein et al., 1981; Williams and Fleming, 1989; Lambert et al., 1987; van Gorkom and Hall, 1968). Infrared (IR) spectroscopy was measured on a Nicolet 7199 FT-IR spectrometer. Mass spectra (MS) were obtained using a Kratos AEI-MS 50 (high resolution, electron impact ionization

EI-MS) for exact mass determinations, MS-12 for chemical ionization (CI-MS) and MS-9 for fast atom bombardment with argon (POSFAB-MS). Microanalyses were measured using a Perkin-Elmer 240 CHN analyzer. All NMR, IR, MS and elemental analyses were performed by Chemistry Services at the University of Alberta.

Flash chromatography was performed according to Still et al. (1978) using silica gel, 70-230 mesh, from Aldrich. Thin layer chromatography (TLC) was used to determine the extent of reactions where possible. Spots were visualized using UV absorption or I_2 staining where applicable. Bromocresol green spray (0.04% in ethanol, made blue by NaOH) was used for visualization of acids. TLC plates used were PE SIL G/UV (20 x 20 cm) with polyester backing purchased from Whatman Ltd. Preparative TLC was performed on PLC silica gel 60 F_{254} S (20 x 20 cm) from Merck.

MOISTURE DETERMINATION IN HONEY.

The moisture content of honey was determined using an Abbe refractometer maintained at 20 °C using a constant temperature bath. The refractive index measurements were converted to moisture content using literature tables (Wedmore, 1955; Crane, 1975; AOAC, 1990b).

HPLC ANALYSIS OF CARBOHYDRATES.

Apparatus: *HPLC column*: (a) 300 x 7.8 mm Aminex HPX-87P (BioRad), 85 °C (BioRad HPLC column heater), deionized distilled water as eluent, guard column: 40 x 4.6 mm Carbo-P microguard cartridge (BioRad). (b) 300 x 7.8 mm Rezex H^+ (Phenomenex), room temperature, 0.01 N H_2SO_4 as eluent, guard column: 40 x 4.6 mm Cation-H microguard cartridge (BioRad). *Pump*: Scientific systems Inc. 210, flow rate 0.6 mL/min. *Injector*: Rheodyne 20 µL loop. *Detector*: Waters differential refractometer R401, 8 x at 25 °C. *Integrator*: Varian 4270.

Sample Preparation: The honey samples studied were provided by BeeMaid (Edmonton, Alberta). Solutions for HPLC analysis were prepared using distilled deionized water containing 1 g/L sodium azide (to prevent enzymatic or bacterial changes in the solution). For analysis on column (a), mixed sugar standards containing 0.302 g sucrose, 1.508 g glucose and 1.902 g fructose were dissolved in the azide-containing water to a total volume of 50 mL. Honey solutions were prepared using 5 g samples in 50 mL of the same solvent. For column (b), the same amount of sugars or honey were dissolved in 100 mL. Before injection into the column, all solutions were filtered through a Sep-Pak C_{18} cartridge (Millipore) then passed through a Millipore Swinney-25 syringe adapter containing 0.45 μm cellulose nitrate paper (Millipore).

HPLC ANALYSIS OF FUMAGILLIN.

The honey (5.0 g) was dissolved in water (25 mL) and the total volume was made up to 50 mL using acetonitrile. The solution was filtered through a Millipore Swinney-25 syringe adapter containing Durapore (polyvinylidene difluoride) membrane filters (Millipore) and immediately injected through a 20 μL loop for analysis on a reversed-phase silica column (Phenomenex IB-SIL 5 C_{18} 150 x 4.6 mm) which was preceded by a guard column (Phenomenex IB-SIL 5 C_{18} 50 x 2.1 mm) and operated at ambient temperature. The mobile phase was acetonitrile : water : glacial acetic acid (500 : 500 : 1.5 v/v/v) pumped by a Scientific Systems Inc. SSI model 300 at a flow rate of 1.0 mL/min, and detection was achieved using a UV detector (BioRad UV monitor model 1305) at 350 nm. The output was recorded on a Hewlett Packard 3388A integrator.

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR FUMAGILLIN.

Preparation of buffer solutions. *Phosphate buffer (pH 7.6)* was prepared by addition of 43.5 mL of 0.2 M disodium hydrogen phosphate (Na_2HPO_4) to 6.5 mL of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$). The solution was made up

to a volume of 100 mL with water. *Phosphate buffered saline (PBS)* solution contained sodium chloride (18.0 g), Na_2HPO_4 (2.22 g), potassium dihydrogen phosphate (KH_2PO_4, 0.6 g) and thimerosal (0.2 g). These were dissolved in 1.9 L water and the pH adjusted to 7.3. The total volume was then made up to 2.0 L with water. To prepare PBST, Tween 20 (1.0 g) was added before the final step. *Citrate buffer* was made up of 0.1 M citric acid monohydrate (23 mL) and 0.1 M sodium citrate dihydrate (27 mL). The pH was adjusted to 4.75 and the resulting solution was diluted with an equal volume of water.

Immunization. Two male rabbits (Flemish giant x French Lop Ear), both 4 weeks old, were injected (each 2 x 0.25 mL subscapularly and 0.5 mL gluteal) with the conjugate LPH-FA (1.0 mg) suspended in sterile PBS (1.0 mL) and emulsified with Freund's complete adjuvant (1.0 mL). Boostings (containing LPH-FA (1.0 mg) in PBS (1.0 mL) with 1.0 mL Freund's incomplete adjuvant) were performed after 3 weeks and then again after 4 weeks. A test bleed at this stage revealed that the titre was low. Therefore, additional boostings were made after 4 weeks and then again after 3 weeks. Four weeks later, a final bleed out was performed (17 weeks after initial immunization). The blood was left at room temperature for about 2 h to coagulate and then it was centrifuged at 1000 rpm for 5 minutes. The resulting clear yellowish serum was stored in 1.5 mL sealed containers at - 20 °C.

Preparation of honey samples for ELISA. To prepare standard solutions of fumagillin in honey, a honey sample was obtained from a producer who does not use any chemicals or medications for his bees, including fumagillin. This standard honey (25.278 g) was dissolved in 50 mL of water. Aliquots of the solution (5.0 mL) were placed in test tubes. To one of the test tubes was added 100 µL of ethanol : water (1 : 4) and used as a control. Fumagillin dicyclohexylamine salt (0.30 mg, equivalent to 0.22 mg fumagillin) was dissolved in 1.0 mL of 95% ethanol, then 4.0 mL of water were added. Several dilutions of the fumagillin solution were made using ethanol : water (1 : 4) as solvent. To each test tube containing honey solution was added 100 µL of one of the diluted fumagillin

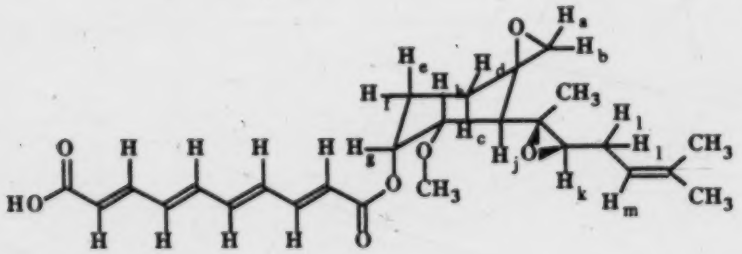
solutions to make up solutions of different levels of fumagillin in honey. To analyze unknown honey samples, 5.0 g of the honey were dissolved in 10.0 mL of water and then 200 µL of ethanol : water (1 : 4) were added.

Indirect competitive ELISA procedure. The procedure of Sheth and Sporns (1990) was applied. The 96 wells on each ELISA microtitre plate were coated with the conjugate BSA-FA in PBS (1.0 µg/mL; 200 µL/well) or blank PBS solution. The plates were stored overnight at 4 °C in a plastic bag containing a wet paper towel. They were then flipped and shaken to remove their contents and 1% BSA in PBS was added to all wells (200 µL/well). The plates were incubated in their plastic bags at room temperature for 1 h. The solutions in the wells were then shaken out and replaced by PBST for washing (3 x 200 µL/well). Test samples, standards or blanks were added to the wells (100 µL/well) and incubated with serum diluted 500 or 1000 times using 0.05 % BSA in PBST (100 µL/well). After being left for 2 h at room temperature in the plastic bags, the plates were again emptied of their contents and washed 3 times with PBST (200 µL/well). Goat antirabbit antibody conjugated to horseradish peroxidase was diluted 3000 times with PBST and added to each well (200 µL/well). The plates were incubated at room temperature for 2 h in the plastic bags and then washed with PBST (3 x 200 µL/well). A solution of the substrates *o*-phenylenediamine (0.4 mg/mL) and urea peroxide (1 mg/mL) in 0.1 M citrate buffer (pH 4.75) was added to each well (200 µL/well). The plates were allowed to remain at room temperature for exactly 30 minutes and then an ELISA reader was used to measure the difference in absorbance ($A_{450\ nm} - A_{660\ nm}$) in each well. The maximum % relative standard deviation of three replicates on a plate was 12 % (average % relative standard deviation for all analyses performed was 6%).

SYNTHESIS.

Fumagillin (1). *2,4,6,8-Decatetraenedioic acid mono-[5-methoxy-4-[2-methyl-3-(3-methyl-2-bytenyl)-oxiranyl]-1-oxaspiro[2.5]oct-6-yl] ester.* Fumagillin

dicyclohexylamine salt (1.46 g, 2.28 mmol) was suspended in water (200 mL) and acidified to pH 4 using dilute acetic acid. The solution was extracted with chloroform (3 x 50 mL) and the combined chloroform layers were washed with water (2 x 50 mL). The chloroform layer was dried (Na_2SO_4) and the solvent was removed under vacuum at 30 °C to give a yellow oil. Recrystallization from 2-pentanone gave fumagillin (1.03 g, 98%) as a white powder: mp 187-188 °C (lit. mp 194-195 °C; Tarbell et al., 1955); IR (CH_2Cl_2 cast) 3240-2980, 1710, 1627, 1124, 1010 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.33 (m, 2H, -C=C*H*), 6.61 (m, 2H, -C=C*H*), 6.45 (m, 2H, -C=C*H*), 6.00 (q, 2H, 3.5 Hz, 15 Hz, -C=C*H*), 5.74 (s, 1H, H_g), 5.23 (t, 1H, 7.5 Hz, H_m), 3.73 (dd, 1H, 3 Hz, 11 Hz, H_h), 3.48 (s, 3H, -OC*H*$_3$), 3.04 (d, 1H, 4.5 Hz, H_a), 2.68 (t, 1H, 6 Hz, H_k), 2.59 (d, 1H, 4.5 Hz, H_b), 2.42 (m, 1H, H_l), 2.18 (m, 2H, H_j, H_l), 2.04 (m, 2H, H_e, H_f), 1.90 (t, 1H, 14 Hz, H_c), 1.78 (s, 3H, -C=C-C*H*$_3$), 1.70 (s, 3H, -C=C-C*H*$_3$), 1.26 (s, 3H, -O-C-C*H*$_3$), 1.12 (d, 1H, 13 Hz, H_d); exact mass 458.2303 (458.2305 calcd for $C_{26}H_{34}O_7$); Anal. Calcd for $C_{26}H_{34}O_7$: C, 68.10; H, 7.47. Found: C, 68.01; H, 7.87.



1

Fumagillol (2) and 2,4,6,8-decatetraenedioic acid (3). Fumagillin (299.1 mg, 0.652 mmol) was dissolved in 0.1 N NaOH (16 mL). The yellow solution obtained was stirred for 4 h at 25 °C. Flocculent crystals formed were filtered and the filterate was extracted with ether (3 x 15 mL). The ether layer was dried over Na_2SO_4 and

the solvent removed under vacuum. A yellow oil was obtained from which the alcohol fumagillol (**2**) was crystallized using petroleum ether (122 mg, 66 %): mp 49-51 °C (lit. mp 54-56 °C; Landquist, 1956); IR (CH_2Cl_2 cast) 3400-3500, 2976, 2928, 2885, 2824, 1445, 1378, 1103 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 5.25 (t, 1H, 7.53 Hz), 4.45 (br, 1H, -OH), 3.72 (m, 1H), 3.58 (s, 3H), 3.04 (d, 1H, 4.5 Hz), 2.68 (t, 1H, 6.5 Hz), 2.64 (d, 1H, 4.5 Hz), 2.46 (m, 2H). 2.28 (m, 2H), 2.60 (m, 1H), 1.86 (s, 3H), 1.76 (s, 3H), 1.33 (s, 3H), 1.10 (m, 1H); CI-MS base peak 283 (MH^+). The yellow aqueous solution was acidified with acetic acid yielding a greenish yellow oily precipitate which was dried over P_2O_5 to give 2,4,6,8-decatetraenedioic acid (**3**) as a yellow powder: mp 293-295 °C (lit. mp 295-297 °C; Schenck et al., 1953); IR (KBr) 3420, 3000-2830, 1672, 1620, 1422, 1308 cm^{-1}; ^1H NMR (360 MHz, DMSO d_6) δ 7.27 (q, 2H, 11Hz, 14.5 Hz), 6.84 (m, 2H), 6.65 (m, 2H), 6.04 (d, 2H, 14,5 Hz); exact mass 194.0578 (194.0579 calcd for $C_{10}H_{10}O_4$).

Fumagillyl hemisuccinate (4). Fumagillol (21.9 mg, 0.078 mmol), succinic anhydride (41.4 mg, 0.411 mmol) and dimethylaminopyridine (2.0 mg, 0.016 mmol) were dissolved in anhydrous pyridine (1.0 mL) and stirred at 22 °C for 3 days. The solvent was removed under vacuum and the resulting residue was dissolved in chloroform (15 mL) and extracted with acidified water (pH 3.5, 3 x 15 mL). The combined water layers were washed with chloroform and the combined chloroform layers were dried (Na_2SO_4). The solvent was removed under vacuum to give a yellow oil (25.4 mg, 85 %): IR (CH_2Cl_2 cast) 2929, 1734, 1160, 1105; exact mass 382.1978 (382.1992 calcd for $C_{20}H_{30}O_7$).

BSA-Fumagillin conjugate (BSA-FA). Fumagillin (5.9 mg, 13 μmol), dicyclohexylcarbodiimide (DCC, 6.1 mg, 30 μmol) and N-hydroxysuccinimide (2.6 mg, 23 μmol) were dissolved in anhydrous dimethylformamide (DMF, 0.1 mL). The solution was stirred at 4 °C for 24 h. The solution was filtered (to remove the needle-like crystals due to dicyclohexylurea) directly into a solution of bovine serum albumin (BSA, 110.5 mg, 1.67 μmol) in 2 mL phosphate buffer solution (pH 7.6). A cloudy solution was obtained

which was stirred at 4 °C for 24 h. The suspension was then transferred to a dialysis bag and dialysed for 24 h in each of the following solutions successively: 8 M urea (1 L), 50 mM ammonium bicarbonate (4 L), 25 mM ammonium bicarbonate (4 L). The dialysed solution was freeze-dried to give the BSA-Fumagillin conjugate (BSA-FA) as an off-white flocculent powder. Anal. found for pure BSA: C, 51.90; H, 7.09; N, 15.77. Anal. found for BSA-FA conjugate: C, 50.50; H, 7.07; N, 15.04. UV absorption for the conjugate (37 μg/mL of PBS) at 350 nm was 0.0755. Using the standard curve for absorption of pure fumagillin (Figure 9), this indicates that the conjugate contains approximately 2 fumagillin groups per BSA molecule.

LPH-Fumagillin conjugate (LPH-FA). Fumagillin (19.3 mg, 42.1 μmol), DCC (13.8 mg, 66.9 μmol), N-hydroxysuccinimide (11.8 mg, 102.5 μmol) were dissolved in anhydrous DMF (2 mL) and stirred at 4 °C for 24 h. The solution was filtered into 2 mL PBS (pH 7.6) containing LPH (84.1 mg, 1.27 μmol). A thick precipitate was formed which was stirred at 4 °C for 24 h. The resulting suspension was dialysed (as for BSA-FA conjugate) and then freeze-dried. Anal. found for pure LPH: C, 49.00; H, 6.72; N, 15.76. Found for LPH-FA conjugate: C, 52.79; H, 7.03; N, 13.42. Therefore, the LPH-FA conjugate contains approximately 18 fumagillin molecules per LPH molecule.

BSA-Fumagillyl hemisuccinate conjugate (BSA-FS). Fumagillyl hemisuccinate (**4**, 25.4 mg, 0.07 mmol), dicyclohexylcarbodiimide (28.5 mg, 0.14 mmol) and N-hydroxysuccinimide (16.7 mg, 0.15 mmol) were dissolved in anhydrous DMF (2.0 mL) and stirred at 4 °C for 24 h. The reaction mixture was filtered into a solution of BSA (306 mg, 4.6 μmol) in PBS (pH 7.6, 2.0 mL) and stirred at 4 °C for 24 h. A thick white precipitate formed immediately after addition of the active ester solution to the solution of BSA, and remained after dialysis (performed as for the BSA-FA conjugate). After freeze-drying, the conjugate BSA-FS was obtained in the form of flaky solid: Anal. found for BSA-FS: C, 49.41; H, 6.72; N, 14.40. Therefore the conjugate has about 14 hapten molecules per BSA.

PRODUCTS OF THE DECOMPOSITION OF FUMAGILLIN.

Decomposition of fumagillin at 60 °C at pH 3.5. Fumagillin (362 mg) was dissolved in 10% ethanol in water (200 mL) and protected from light. The pH of the solution was lowered to 3.5 using dilute acetic acid and the solution was stirred at 60 °C for 42 days. The solvent was then removed under vacuum and the resulting compound was dissolved in acidified water (pH 3.5) and extracted with dichloromethane. The dichloromethane layer was collected and the solvent removed under vacuum. The resulting oil was purified by flash chromatography using ethyl acetate : hexane (2 : 1). Two main compounds were isolated, one of which was found to be unreacted fumagillin. The second compound (**5**) was isolated as a white powder after recrystallization from ether: mp 161-163 °C; IR (KBr) 3431, 2940, 1710, 1628, 1277, 1237 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$-DMSO d_6) δ 7.30 (m, 2H, 4.5 Hz, 13 Hz), 6.63 (m, 2H), 6.50 (m, 2H), 5.97 (q, 2H, 11.5 Hz, 15 Hz), 5.65 (d, 1H, 3 Hz), 5.32 (m, 1H), 4.29 (br, 1H, -O*H*), 3.87 (d, 1H, 9 Hz), 3.80 (br, 1H, -O*H*), 3.73 (dd, 1H, 3 Hz, 12 Hz), 3.62 (d, 1H, 9 Hz), 3.39 (s, 3H), 3.32 (q, 1H, 2 Hz, 11 Hz), 2.41 (q, 1H, 7 Hz, 14 Hz), 2.11 (m, 3H), 1.89 (m, 1H, 14 Hz), 1.72 (s, 3H), 1.71 (m, 1H), 1.64 (s, 3H), 1.61 (d, 1H, 4 Hz), 1.32 (s, 3H); EI-MS 407, 389, 345, 327, 295, 283, 257, base peak 151; CI-MS 450, 433, 318, 301, 283, 268, 251, 233, base peak at 35; POSFAB-MS (cleland) 477 (MH^+), 499 (M+23); Anal. Calcd for $C_{26}H_{36}O_8$: C, 65.53; H, 7.61. Found: C, 65.31; H, 7.42.

Decomposition of fumagillin in light. Fumagillin (180 mg) was dissolved in acetonitrile : water (1 : 1, 10 mL) and placed under sunlight/fluorescent light at room temperature. After 7 days, TLC indicated complete decomposition of fumagillin. The solvent was removed under vacuum and the residue was dissolved in chloroform (40 mL) and washed with water (3 x 20 mL). The chloroform layer was then dried (Na_2SO_4) and the solvent was removed under vacuum to give a yellow oil. Attempts to purify this product further by recrystallization led to some decomposition. Preparative TLC using toluene : methanol 3 : 1 gave **6** as a reddish oil: IR ($CHCl_3$ cast) 3260, 2951, 2927, 2860, 2659,

2250, 2124, 1710, 1617, 1480 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.17 (m, 1H), 6.39 (m, 1H), 6.16 (m, 2H), 5.77 (m, 2H), 5.56 (s, 1H), 5.09 (t, 1H, 6 Hz), 3.55 (m, 1H), 3.28 (s, 3H), 2.85 (d, 1H, 4.5 Hz), 2.51 (t, 1H, 6 Hz), 2.48 (d, 1H, 4 Hz), 2.25 (m, 1H), 2.05 (m, 2H), 1.89 (m, 3H), 1.77 (m, 2H), 1.66 (s, 3H), 1.52 (s, 3H), 1.05 (s, 3H), 0.98 (d, 1H, 13 Hz); EI-MS 413, 282, 231; CI-MS 666, 531, 503, 417, 373, 300, 283, 265 base peak 35; POSFAB-MS (cleland) 459 (MH^+), 481 (M+23); UV absorption maxima at 240 and 306 nm.

EXPERIMENTS TO DETERMINE THE RATE OF DECOMPOSITION OF FUMAGILLIN IN HONEY.

EXP. A - decomposition in the dark at 37 °C. A honey sample (25.807 g) was weighed out in a round bottomed flask. Fumagillin dicyclohexylamine (FDCH, 0.048 g, equivalent to 0.035 g fumagillin) was dissolved in 95% ethanol (0.5 mL) and 1.2 mL water was added. The fumagillin solution was added to the honey solution and stirred. The flask was sealed, protected from light and placed in an oil bath maintained at 37 °C. A control sample of the honey (25.298 g), to which 95% ethanol (0.5 mL) and water (1.2 mL) had been added, was also placed in the same oil bath and subjected to the same conditions as the above sanple.

EXP. B - decomposition in the dark at 80 °C. A flask containing a sample of the honey (25.236 g) and FDCH (0.034 g, equivalent to 0.024 g fumagillin) dissolved in 95% ethanol (1.0 mL) and water (1.2 mL) was protected from light and stirred in an oil bath at 80 °C. The control sample contained honey (24.779 g), 95% ethanol (1.0 mL) and water (1.2 mL).

EXP. C - decomposition in light at 37 °C. A sample of the same honey (25.969 g) was weighed out in a round bottomed flask. FDCH (0.050 g, equivalent to 0.034 g fumagillin) was dissolved in 95% ethanol (0.5 mL) and 1.2 mL water was added. This solution was added to the honey and the flask was sealed and stirred in an oil bath

maintained at 37 °C. Light was directed to the sample (Elmo S-30 projector with a high efficiency projection lamp 120 V, 300 watt, 30 cm away from the sample). Another flask containing another sample of the same honey (25.503 g) was used as a control. Ethanol (95%, 0.7 mL) and water (1.0 mL) were added to it and it was placed in the same oil bath as the sample containing fumagillin.

CHAPTER 6

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